Postprint of Strain Screening for Efficient Degradation of Free Gossypol and Improvement of Nutritional Quality of Cottonseed Meal

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Abstract

This study aimed to utilize *Bacillus subtilis* and *Lactobacillus plantarum* to ferment cottonseed meal, investigating their effects on the degradation rate of free gossypol in cottonseed meal and comparing nutritional quality indicators such as viable count, neutral protease activity, acid-soluble protein content, pH, and free gossypol content before and after fermentation. The results revealed that the *Bacillus subtilis* strain capable of efficiently degrading free gossypol in cottonseed meal was BLCC1-0039, and the *Lactobacillus plantarum* strain that could effectively improve fermentation flavor was BLCC2-0092. By integrating the advantages of these two probiotic strains, the optimal combined fermentation method was identified as inoculating *Lactobacillus plantarum* BLCC2-0092 and *Bacillus subtilis* BLCC1-0039 at a 1:1 ratio, followed by aerobic fermentation at 37 °C for 24 h and subsequent anaerobic fermentation. Compared with the blank control group, the pH of fermented cottonseed meal at each fermentation stage in the optimal combined fermentation group was significantly decreased ($P < 0.05$), dropping to 5.27 at 72 h of anaerobic fermentation; the acid-soluble protein content was significantly increased ($P < 0.05$), reaching 23.54% at 72 h of anaerobic fermentation; and the free gossypol content was significantly decreased ($P < 0.05$), with a degradation rate of 52.12% at 24 h of aerobic fermentation and 61.58% at 72 h of anaerobic fermentation. These findings demonstrate that combined fermentation with *Lactobacillus plantarum* BLCC2-0092 and *Bacillus subtilis* BLCC1-0039 can effectively reduce free gossypol content and improve the nutritional quality of fermented cottonseed meal.

Full Text

Screening of Highly Efficient Strains for Degrading Free Gossypol and Improving the Nutritional Quality of Cottonseed Meal

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Abstract

This study investigated the effects of fermenting cottonseed meal with *Bacillus subtilis* and *Lactobacillus plantarum* on free gossypol degradation and compared key nutritional quality indicators before and after fermentation, including viable bacterial counts, neutral protease activity, acid-soluble protein content, pH, and free gossypol content. The results identified *Bacillus subtilis* strain BLCC1-0039 as highly effective at degrading free gossypol in cottonseed meal, while *Lactobacillus plantarum* strain BLCC2-0092 significantly improved fermentation flavor. Combining the advantages of both probiotics, the optimal co-fermentation conditions were determined to be a 1:1 inoculation ratio of *Lactobacillus plantarum* BLCC2-0092 and *Bacillus subtilis* BLCC1-0039, with 24 hours of aerobic fermentation followed by anaerobic fermentation at 37 °C. Compared with the blank control group, the optimal co-fermentation group exhibited significantly reduced pH values at all fermentation stages ($P < 0.05$), with pH dropping to 5.27 after 72 hours of anaerobic fermentation. Acid-soluble protein content increased significantly ($P < 0.05$), reaching 23.54% after 72 hours of anaerobic fermentation. Free gossypol content decreased significantly ($P < 0.05$), with degradation rates of 52.12% after 24 hours of aerobic fermentation and 61.58% after 72 hours of anaerobic fermentation. These findings demonstrate that co-fermentation with *Lactobacillus plantarum* BLCC2-0092 and *Bacillus subtilis* BLCC1-0039 effectively reduces free gossypol content while improving the nutritional quality of fermented cottonseed meal.

Keywords: *Bacillus subtilis*; *Lactobacillus plantarum*; cottonseed meal; free gossypol; nutritional quality

Introduction

With the rapid development of animal husbandry in China, feed demand continues to grow. Cottonseed cake (meal) represents a high-quality plant protein feed, containing 20–40% crude protein and approximately 11% crude fiber, along with substantial thiamine and organic phosphorus content, making it a valuable feed ingredient for livestock and poultry diets. However, cottonseed meal contains toxic substances including gossypol, cyclopropenoid fatty acids, and tannins that limit its application in animal production, with gossypol being the most

problematic. Gossypol exists in two forms: bound and free. Bound gossypol passes through the digestive system unabsorbed and is rapidly excreted in feces, exhibiting low toxicity. In contrast, the active groups (aldehyde and carboxyl groups) in free gossypol molecules are highly toxic to animals. Long-term feeding of diets containing excessive untreated cottonseed meal leads to gossypol accumulation in animal tissues, causing poisoning characterized by acute respiratory distress, anorexia, fatigue, and even death.

Primary detoxification methods for cottonseed meal include physical, chemical, solvent extraction, and microbial fermentation approaches, with microbial fermentation showing the greatest application potential. This method not only removes free gossypol but also increases crude protein content while retaining enzymes, vitamins, amino acids, and growth-promoting factors in the fermentation substrate, substantially improving nutritional and feeding value and enhancing utilization efficiency. However, different microbial strains exhibit varying capacities for gossypol degradation, making strain selection the critical first step in ensuring effective detoxification and nutritional improvement. Previous reports indicate that *Bacillus* species demonstrate high gossypol-degrading activity, though some strains produce pungent ammonia through amino acid decarboxylation or deamination, negatively affecting fermentation aroma. *Lactobacillus* species also show high gossypol-degrading activity while producing lactic acid, acetic acid, and other volatile compounds that improve feed palatability. This study aimed to utilize *Bacillus subtilis* and *Lactobacillus plantarum* for solid-state fermentation of cottonseed meal to reduce gossypol content and enhance nutritional value.

Materials and Methods

1.1 Fermentation Materials and Strains

Cottonseed meal used in the experiments was provided by Xinjiang Tai Kun Biological Group and ground to pass through a 40-mesh sieve. *Bacillus subtilis* strains included BLCC1-0039, BLCC1-0090, BLCC1-0157, and BLCC1-0169. *Lactobacillus plantarum* strains included BLCC2-0015, BLCC2-0092, BLCC2-0111, BLCC2-0112, and BLCC2-0111. All strains were preserved in the Culture Collection Center of the Research Institute at Shandong Baolai-Leelai Bioengineering Co., Ltd.

1.2 Culture Media

Yeast Extract Peptone Medium: 2 g glucose, 10 g peptone, 5 g sodium chloride, and 5 g yeast extract dissolved in distilled water to 1000 mL, pH adjusted to 7.0, sterilized at 121 °C for 20 min.

MRS Medium: 20 g glucose, 10 g peptone, 8 g beef extract, 4 g yeast extract, 0.5 g magnesium sulfate, 0.3 g manganese sulfate, 2 g ammonium citrate, 5 g

sodium acetate, and 1 mL Tween-80 dissolved in distilled water to 1000 mL, pH adjusted to 6.0, sterilized at 121 °C for 20 min.

1.3 Chemical Reagents

Glucose (Shandong Xiangrui Pharmaceutical Co., Ltd.), peptone (Beijing Aoboxing Biotechnology Co., Ltd.), sodium chloride (analytical pure, Tianjin Bodi Chemical Co., Ltd.), yeast extract and beef extract (Tianjin Yingbo Biochemical Reagent Co., Ltd.), magnesium sulfate and manganese sulfate (Jinan Huifengda Chemical Co., Ltd.), ammonium citrate (Shanghai Fusheng Industrial Co., Ltd.), sodium acetate (Qingdao Jieshikang Biotechnology Co., Ltd.), Tween-80 (Tianjin Kaitong Chemical Co., Ltd.), acetonitrile, acetone, and methanol (chromatographic grade, Tianjin Yongda Chemical Co., Ltd.), phosphoric acid (analytical pure, Tianjin Kaitong Chemical Co., Ltd.), microporous filter membranes (13 mm diameter, 0.22 μm pore size, Shanghai Anpu Co., Ltd.), and gossypol standard (HPLC grade, Shanghai Yuanye Biotechnology Co., Ltd.).

1.4 Experimental Procedures

1.4.1 Seed Culture Preparation A loopful (approximately 0.05 g) of twice-activated *Bacillus subtilis* slant culture was inoculated into a 500 mL flask containing 100 mL yeast extract peptone medium and incubated at 37 °C with shaking at 180 rpm for 24 h. A loopful (approximately 0.05 g) of twice-activated *Lactobacillus plantarum* slant culture was inoculated into a 500 mL flask containing 100 mL MRS medium and incubated statically at 37 °C for 24 h.

1.4.2 Screening of *Bacillus subtilis* Strains Raw material fermentation was employed. Cottonseed meal was weighed into 1000 mL flasks at a material-to-water ratio of 1.0:0.4 (g:mL) with 100 g per flask. Prepared *Bacillus subtilis* seed cultures were inoculated at 2% concentration, with three replicates per sample. Blank controls without bacterial inoculation were included. All flasks were incubated at 37 °C for aerobic fermentation. Samples were collected at 24 and 48 h to determine viable *Bacillus* counts, neutral protease activity, acid-soluble protein content, and free gossypol content.

1.4.3 Screening of *Lactobacillus plantarum* Strains Raw material fermentation was employed. Cottonseed meal was weighed into bags at a material-to-water ratio of 1.0:0.4 (g:mL) with 100 g per bag. Prepared *Lactobacillus plantarum* seed cultures were inoculated at 2% concentration, sealed, with three replicates per sample. Blank controls without bacterial inoculation were included. All bags were incubated at 37 °C for anaerobic fermentation. Samples were collected at 24 and 48 h to determine viable *Lactobacillus* counts, pH, acid-soluble protein content, and free gossypol content.

1.4.4 First Co-fermentation of *Bacillus subtilis* and *Lactobacillus plantarum* Selected *Bacillus subtilis* and *Lactobacillus plantarum* strains were co-fermented using a sequential aerobic (7 h) then anaerobic (48 h) process. Cottonseed meal was weighed into bags at a material-to-water ratio of 1.0:0.4 (g:mL) with 100 g per bag. The mixed inoculum (1:1 ratio) was added at 2% concentration, with three replicates per sample. Blank controls without bacterial inoculation were included. All bags were incubated at 37 °C. Samples were collected at 24 and 48 h of anaerobic fermentation to determine viable counts of both *Lactobacillus* and *Bacillus*, pH, and neutral protease activity.

1.4.5 Second Co-fermentation of *Bacillus subtilis* and *Lactobacillus plantarum* Based on first-round results, the fermentation protocol was modified to extend aerobic fermentation to 24 h followed by 48 h anaerobic fermentation (total 72 h). Cottonseed meal was weighed into bags at a material-to-water ratio of 1.0:0.4 (g:mL) with 100 g per bag. The mixed inoculum (1:1 ratio) was added at 2% concentration, with three replicates per sample. Blank controls without bacterial inoculation were included. All bags were incubated at 37 °C. Samples were collected at 24 h of aerobic fermentation and at 48 and 72 h of anaerobic fermentation to determine viable counts of both microorganisms, neutral protease activity, pH, acid-soluble protein content, and free gossypol content.

1.5 Analytical Methods

1.5.1 Viable Count Determination Ten grams of fermented cottonseed meal was accurately weighed and dispersed with glass beads for dilution counting. Ten-fold serial dilutions were prepared with physiological saline. Appropriate dilutions were plated on yeast extract peptone medium for *Bacillus* counts and MRS medium for *Lactobacillus* counts. Plates were incubated at 37 °C for 48 h, and colony counts were used to calculate viable counts expressed as CFU/g.

1.5.2 Neutral Protease Activity Assay One gram of fermented cottonseed meal was accurately weighed and dissolved in 8 volumes of phosphate buffer (pH 7.5). After thorough mixing and centrifugation, the supernatant was collected. Neutral protease activity was determined using the Folin-phenol reagent method. One unit of neutral protease activity was defined as the amount of enzyme that hydrolyzes casein to produce 1 μg of tyrosine per minute under specified temperature and pH conditions.

1.5.3 Acid-Soluble Protein Content Determination Two grams of fermented cottonseed meal was accurately weighed into a 25 mL stoppered test tube, mixed with 10 mL of 15% trichloroacetic acid solution, and allowed to stand for 5 min before dilution to 25 mL. The mixture was blended every 2 min for a total of 30 min, then quantitatively transferred and filtered. Ten milliliters

of filtrate was transferred to a digestion tube, mixed with 3 g of mixed catalyst (potassium sulfate:anhydrous copper sulfate = 15:1), followed by addition of 7-8 mL concentrated sulfuric acid. The tube was digested at 420 °C until the solution became clear blue-green, then digestion continued for an additional 40 min. Soluble protein content in the supernatant was determined by the Kjeldahl method. Crude protein content in the original sample was determined separately using the Kjeldahl method. Acid-soluble protein content (%) was calculated as: (soluble protein content in supernatant × 25/10) / crude protein content in sample.

1.5.4 Free Gossypol Content Determination Preparation of Gossypol Standard Solution: Gossypol standard was accurately weighed and dissolved in acetonitrile-0.2% phosphoric acid (85:15, v/v) to prepare a 1.21 mg/mL stock solution. The stock solution was further diluted with the same solvent to obtain a 121 μg/mL working solution. This working solution was serially diluted with mobile phase to concentrations of 61.50, 20.17, 5.04, 2.52, and 1.21 μg/mL. Samples were injected from low to high concentration, and a calibration curve was constructed using peak area versus concentration. The regression equation was $Y = 0.00000545X + 0.160525$ ($R^2 = 0.9999652$) with a linear range of 1.21-121 μg/mL.

Extraction of Free Gossypol from Samples: Three grams of sample was accurately weighed, mixed with 30 mL acetone, and ultrasonically extracted for 30 min. After centrifugation at 3000 rpm for 10 min at 25 °C, the supernatant was collected. The extraction was repeated three times, and supernatants were combined. The combined supernatant was transferred to an evaporation flask and rotary-evaporated to dryness. The residue was dissolved in acetonitrile-0.2% phosphoric acid (85:15, v/v), ultrasonically washed, transferred to a 25 mL volumetric flask, diluted to volume, and filtered through a 0.22 μm membrane for HPLC analysis.

HPLC Conditions: Column: Inertsil® ODS-2 (150 mm × 4.6 mm, 5 μm); Mobile phase: acetonitrile-0.2% phosphoric acid (85:15, v/v); Flow rate: 1.0 mL/min; UV detection wavelength: 235 nm; Injection volume: 20 μL; Column temperature: 25 °C.

1.6 Statistical Analysis

Experimental data were preliminarily processed using Excel 2007 and statistically analyzed using SPSS 13.0 software. One-way ANOVA was performed for variance analysis, and LSD test was used for multiple comparisons between groups. Results were expressed as "mean ± standard deviation," with $P < 0.05$ indicating significant differences.

Results

2.1 Gossypol Standard Curve

The HPLC chromatogram of gossypol standard (Figure 1 [Figure 1: see original paper], gossypol concentration 6.15 μg/mL) showed a stable, well-resolved peak at approximately 5.686 min with ideal peak shape and high sensitivity. The standard curve (Figure 2 [Figure 2: see original paper]) demonstrated excellent linearity, wide quantitative range, low detection limit, and high accuracy and sensitivity, confirming the method' s suitability for accurate free gossypol quantification.

2.2 Screening of *Bacillus subtilis* Strains

At 24 h fermentation, all *Bacillus* strains successfully fermented cottonseed meal, achieving viable counts in the billions per gram. Strains BLCC1-0039 and BLCC1-0157 showed significantly higher counts than BLCC1-0090 and BLCC1-0169 ($P < 0.05$). Acid-soluble protein content was highest in BLCC1-0039, followed by BLCC1-0157, both significantly higher than the blank control ($P < 0.05$), with BLCC1-0039 showing a 54.80% increase over control. Neutral protease activity was highest in BLCC1-0039 at 4111.40 U/g, followed by BLCC1-0157, both significantly higher than BLCC1-0090 and BLCC1-0169 ($P < 0.05$), though not significantly different from each other.

At 48 h fermentation, viable counts reached billions per gram or higher, with BLCC1-0039 showing the highest counts followed by BLCC1-0157. All strains significantly increased acid-soluble protein content compared to control ($P < 0.05$), with BLCC1-0039 achieving the highest level (69.43% increase over control). Neutral protease activity remained highest in BLCC1-0039 (3983.24 U/g), followed by BLCC1-0157, both significantly higher than control and the other two strains ($P < 0.05$), but not significantly different from each other. These results indicate that all *Bacillus subtilis* strains improved acid-soluble protein content and neutral protease activity, with BLCC1-0039 demonstrating the best overall performance.

Free gossypol analysis (Table 2) revealed that BLCC1-0039 produced the lowest free gossypol content at 24 h, significantly lower than all other groups ($P < 0.05$), with a degradation rate of 93.89%. At 48 h, BLCC1-0039 again showed the lowest free gossypol content, significantly lower than the other three strains ($P < 0.05$), achieving a degradation rate of 96.67%. These findings confirm BLCC1-0039 as the most effective strain for gossypol degradation among the four tested *Bacillus subtilis* isolates.

2.3 Screening of *Lactobacillus plantarum* Strains

Among the five *Lactobacillus* strains (Table 3), viable counts reached billions per gram at 24 h, except for BLCC2-0112 which showed significantly lower counts ($P < 0.05$). At 48 h, BLCC2-0112 again exhibited the lowest viable counts,

significantly lower than other strains ($P < 0.05$). No significant differences were observed among strains for acid-soluble protein content or free gossypol content at either time point. However, pH values were lowest for BLCC2-0092 and BLCC2-0001 at both 24 and 48 h, followed by BLCC2-0015. These three strains reduced pH below 5.00 at 48 h, with BLCC2-0092 achieving the lowest pH of 4.67. Based on pH reduction capability, BLCC2-0092, BLCC2-0001, and BLCC2-0015 performed well, with BLCC2-0092 being the optimal strain.

2.4 First Co-fermentation Trial

In the first co-fermentation experiment with sequential aerobic (7 h) and anaerobic fermentation, all co-cultures achieved viable *Lactobacillus* counts in the billions per gram after 24 h anaerobic fermentation, with pH dropping below 6.00 and further decreasing below 5.00 after 48 h, confirming successful *Lactobacillus* fermentation. *Bacillus* viable counts increased by one order of magnitude between 24 and 48 h anaerobic fermentation, but neutral protease activity remained low, peaking at only 201.18 U/g after 48 h (compared to 3983.24 U/g achieved by BLCC1-0039 alone). These results indicated that the initial fermentation conditions were unsuitable for *Bacillus* growth.

2.5 Second Co-fermentation Trial

Based on the first trial's limitations, the protocol was modified to extend aerobic fermentation to 24 h, using the most effective strains (*Lactobacillus plantarum* BLCC2-0092, BLCC2-0001 and *Bacillus subtilis* BLCC1-0039). The second co-fermentation achieved viable counts in the billions per gram for both microorganisms, with neutral protease activity exceeding 2000 U/g for all co-cultures. The BLCC2-0092 + BLCC1-0039 combination demonstrated the best overall performance. Compared with the blank control, this optimal co-fermentation group showed significantly reduced pH at all stages ($P < 0.05$), dropping to 5.27 after 72 h anaerobic fermentation. Acid-soluble protein content increased significantly ($P < 0.05$), reaching 23.54% after 72 h anaerobic fermentation. Free gossypol content decreased significantly ($P < 0.05$), with degradation rates of 52.12% after 24 h aerobic fermentation and 61.58% after 72 h anaerobic fermentation.

Discussion

Strain selection is critical for microbial detoxification of cottonseed meal, as different microorganisms exhibit varying gossypol-degrading capacities. Optimizing microbial fermentation to reduce free gossypol while simultaneously improving crude protein content and other feeding qualities can increase inclusion rates in animal diets and enhance cottonseed meal utilization. The primary goal of fermenting and enzymatically treating protein meals is to degrade large proteins into smaller molecules, peptides, and oligopeptides, thereby promoting

protein synthesis, mineral absorption, immune function, and animal growth performance. Acid-soluble protein content serves as an effective quality indicator for fermented protein meals, reflecting both the degree of antigenic protein and antinutritional factor hydrolysis and the resulting peptide content.

Bacillus subtilis is a GRAS (Generally Recognized As Safe) microorganism approved by the U.S. Food and Drug Administration and represents a major industrial enzyme producer. Neutral protease activity is a key indicator of *Bacillus* growth and performance. Previous studies have shown that *Bacillus subtilis* fermentation, particularly with papain supplementation, effectively improves cottonseed meal nutritional value. However, reported gossypol-degrading strains have been limited primarily to yeasts and molds, with few studies on natural fungi. Among *Bacillus* strains, *Bacillus subtilis* M-9 and M-4 have demonstrated high gossypol-degrading activity but require additional carbon sources (increasing costs), high inoculation rates (10%), and long degradation times (>60 h). Additionally, *Bacillus cereus* Br effectively degrades gossypol only in sterilized cottonseed meal, as native microorganisms inhibit its growth in non-sterilized substrates.

In this study, *Bacillus subtilis* BLCC1-0039 alone achieved a 93.89% free gossypol degradation rate after 24 h fermentation at 37 °C, while maintaining high neutral protease activity (4111.40 U/g) and acid-soluble protein content (21.10%). Although literature on *Lactobacillus* for gossypol degradation is scarce, *Lactobacillus*-fermented feed offers advantages including pleasant aroma, good palatability, simple application, high digestibility, and stable efficacy. This study found that while *Lactobacillus plantarum* lacked gossypol-degrading capability, it effectively reduced pH, with BLCC2-0092 decreasing pH to 5.47 after 24 h fermentation. Co-fermentation with *Lactobacillus* and *Bacillus* thus simultaneously reduced free gossypol while eliminating the pungent ammonia odor from *Bacillus* metabolism, improving fermentation aroma and nutritional value.

The second co-fermentation trial demonstrated that extended aerobic fermentation (24 h) benefited *Bacillus* growth, which was crucial for gossypol degradation, while subsequent anaerobic fermentation favored *Lactobacillus* growth and pH reduction, essential for flavor improvement. The optimal combination (BLCC2-0092 + BLCC1-0039, 1:1 ratio) significantly reduced pH to 5.27 after 72 h anaerobic fermentation, increased acid-soluble protein content to 23.54%, and achieved gossypol degradation rates of 52.12% (24 h aerobic) and 61.58% (72 h anaerobic). These degradation rates exceed the 48.5% reported by Wang et al. using *Bacillus subtilis* and *Saccharomyces cerevisiae* co-fermentation at 30 °C for 48 h.

Conclusions

1. *Bacillus subtilis* BLCC1-0039 effectively degrades free gossypol in cottonseed meal, achieving a 93.89% degradation rate after 24 h fermentation while significantly increasing neutral protease activity and acid-soluble protein content.
2. *Lactobacillus plantarum* lacks free gossypol-degrading capability but effectively reduces fermentation pH, with strain BLCC2-0092 decreasing pH to 4.67 after 48 h fermentation.
3. Co-fermentation with *Bacillus subtilis* and *Lactobacillus plantarum* effectively reduces free gossypol content, increases neutral protease activity and acid-soluble protein content, and lowers pH to enhance acidic aroma.
4. Based on comprehensive evaluation of all indicators, the optimal co-fermentation protocol is a 1:1 inoculation ratio of *Lactobacillus plantarum* BLCC2-0092 and *Bacillus subtilis* BLCC1-0039, with 24 h aerobic fermentation at 37 °C followed by anaerobic fermentation.

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