Screening of Cellulolytic Bacteria from Giant Panda Feces and Optimization of Enzyme Production Conditions: Postprint

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Abstract

This study aimed to screen for cellulose-degrading bacterial strains from giant panda feces, and to identify the strain and optimize its enzyme production conditions. Using a medium with sodium carboxymethyl cellulose (CMC-Na) as the sole carbon source, combined with iodine staining, filter paper decomposition test, and cellulase activity assay, one cellulose-degrading strain designated DL was isolated from giant panda feces. Based on morphological observation, physiological and biochemical characteristics, and 16S rDNA gene sequence homology analysis, the strain was preliminarily identified as Paenibacillus cookii LZ033, a spore-forming, aerobic Gram-positive bacterium. To determine the optimal enzyme production conditions for strain DL, four factors were selected: initial pH of the medium, cultivation temperature, shaker rotation speed, and liquid loading volume. Based on the results of single-factor experiments, orthogonal experiments were conducted to determine the optimal enzyme production conditions for strain DL as follows: initial pH of the medium at 6, cultivation temperature at 35 °C, shaker rotation speed at 125 r/min, and liquid loading volume of 100 mL in a 250 mL Erlenmeyer flask. Under these conditions, the cellulase activity (expressed as filter paper activity) reached 102.3 U/mL.

Full Text

Screening of Cellulolytic Bacteria from Giant Panda Feces and Optimization of Enzyme Production Conditions

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Abstract: This study aimed to screen cellulose-degrading bacterial strains from giant panda feces, identify the selected strain, and optimize its enzyme production conditions. Using sodium carboxymethylcellulose (CMC-Na) as the sole carbon source, combined with iodine staining, filter paper decomposition tests, and cellulase activity assays, one cellulose-degrading strain designated DL was isolated from giant panda feces. Based on morphological observation, physiological and biochemical characteristics, and 16S rDNA sequence homology analysis, the strain was preliminarily identified as *Paenibacillus cookii* LZ033, a spore-forming, aerobic, Gram-positive bacterium. To determine the optimal enzyme production conditions for strain DL, four factors—medium initial pH, culture temperature, shaker speed, and liquid medium volume—were selected. Building upon single-factor experiment results, orthogonal testing revealed that the optimal enzyme production conditions were: medium initial pH 6, culture temperature 35 °C, shaker speed 125 r/min, and 100 mL medium volume in a 250 mL flask. Under these conditions, cellulase activity (expressed as filter paper activity) reached 102.3 U/mL.

Keywords: giant panda feces; cellulolytic bacterium; enzyme production conditions

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The giant panda is a rare and endemic species in China, listed as one of the world' s ten most endangered species in 1984 [1-2]. Subadult pandas transition to a bamboo-dominated, high-fiber diet at 10 months of age [3], with adult individuals consuming 12–38 kg of bamboo daily [4]. Pandas can utilize only 8% of cellulose and 27% of hemicellulose from bamboo [5]. Their digestive system is typical of carnivorous mammals [6], and the 2010 publication of the giant panda genome sequence revealed genes encoding enzymes associated with carnivorous digestive systems but no cellulase genes [7]. Consequently, cellulose digestion in pandas is primarily accomplished by gut microorganisms [8-10]. The panda gut consists only of small and large intestines, is relatively short, and contains high oxygen levels, suggesting it is more suitable for aerobic or facultative anaerobic microorganisms [11]. Any alteration in the gut microbiota may cause digestive disorders and even death. Therefore, studying the panda' s intestinal microbial flora is crucial for preventing intestinal diseases, improving panda health, and developing microecological preparations. Although many cellulose-degrading microorganisms exist in nature, pandas are endangered animals requiring special safety considerations. Cellulose-degrading bacteria isolated from panda feces are more suitable as microecological preparations or feed additives than microorganisms from other environments.

In recent years, panda gut microbiota has attracted widespread attention. Zhang Zhihe et al. [12] and Hirayama et al. [13] isolated and identified panda gut flora. Jiang Fang [14] isolated *Serratia* species producing cellulase from panda feces, while Gu Wuyang [15] isolated cellulase-producing bacilli. Zhou et al. [16] isolated bacilli from panda intestines that could both decompose cellulose and inhibit pathogenic bacteria. This study aimed to isolate an aerobic

cellulose-degrading bacterium from the feces of a healthy, non-diarrheic panda named Lin Bing at the Ya' an Bifengxia Base, optimize its enzyme production conditions, enrich microbial sources of cellulase, and provide reference data for preparing panda microecological preparations.

1.1 Sample Source

Fresh feces were collected from a healthy, non-diarrheic female giant panda named Lin Bing (born 2009) at the Ya' an Bifengxia Base.

1.2 Culture Media

- **Beef extract peptone medium:** beef extract 3 g, peptone 10 g, NaCl 5 g, distilled water 1000 mL [17].
- **Screening medium:** beef extract 3 g, CMC-Na 4 g, NaCl 5 g, agar 16 g, distilled water 1000 mL.
- **Strain preservation medium:** yeast extract 5 g, peptone 10 g, NaCl 10 g, agar 20 g, distilled water 1000 mL [18].
- **Fermentation medium:** KH PO 1 g, glucose 6 g, peptone 8 g, MgSO 0.5 g, distilled water 1000 mL.

1.3.1 Enrichment Culture

In a laminar flow hood, 10 g of fresh feces (middle portion) was weighed and placed in 90 mL sterile water containing glass beads, then shaken continuously for 20 min to prepare a bacterial suspension. One milliliter of the suspension was added to 100 mL beef extract peptone medium and cultured at 37 °C, 150 r/min for 24 h [6].

1.3.2 Primary Screening

The mixed fecal bacterial culture was subjected to standard 10-fold serial dilution [19]. Aliquots (100 µL) of 10 , 10 , 10 , and 10 dilutions were spread on screening medium plates, with three replicates per dilution. Plates were inverted and incubated at 37 °C for 24 h. After incubation, iodine solution was added for staining [20], left to stand for 3 min, and transparent zones were observed.

1.3.3 Secondary Screening

The diameters of transparent zones (D, cm) and colonies (d, cm) were measured, and their ratio (D/d) was calculated. Strains with larger ratios were selected for cellulase activity measurement [21].

1.3.3.1 Enzyme Activity Assay Cellulase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method [22]. Total cellulase activity is typically expressed as filter paper activity (FPA). FPA is defined as the amount of enzyme

required to produce 1 μmol glucose per hour from the substrate. FPA (U/mL) was calculated as:

FPA (U/mL) = (glucose × enzyme solution volume × 5.56) / (enzyme amount added × filter paper weight × time)

where 5.56 represents the μmol equivalent of 1 mg glucose.

1.3.3.2 Filter Paper Decomposition Test Selected strains were inoculated into liquid medium containing filter paper as the sole carbon source [19], with uninoculated medium as control. Cultures were incubated at 37 °C, 150 r/min for 7 days, with daily photographic documentation of filter paper decomposition.

1.4.1 Morphological and Physiological-Biochemical Characterization

Colony morphology including elevation, shape, transparency, texture, color, and margin was observed. Cellular morphology was examined via Gram staining under microscopy for cell size, structure, arrangement, spores, and flagella [7]. Physiological and biochemical characteristics were determined according to *Taxonomic Outline of the Prokaryotes, Bergey' s Manual of Systematic Bacteriology* [23] and *Manual of Systematic Identification of Common Bacteria* [24].

1.4.2 16S rDNA PCR Amplification and Sequence Analysis

Genomic DNA was extracted using a commercial kit. The 16S rDNA sequence was amplified using primers 7F (5'-CAGAGTTTGATCCTGGCT-3') and 1540R (5' -AGGAGGTGTCCAGCCGCA). PCR conditions were: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 10 min; storage at 4 °C [25]. PCR products were sequenced by Shanghai Sangon Biotech Co., Ltd., and the 16S rDNA sequence was compared against the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

1.5.1 Effect of Medium Initial pH on Enzyme Production

Medium pH was adjusted to 5.5, 6.0, 6.5, 7.0, and 7.5 using 1 mol/L HCl and 1 mol/L NaOH. Four percent inoculum was added to 100 mL medium in 250 mL flasks, cultured at 37 °C, 150 r/min for 24 h, and FPA was measured.

1.5.2 Effect of Culture Temperature on Enzyme Production

Medium initial pH was set at 6.5, 4% inoculum was added to 100 mL medium in 250 mL flasks, and cultures were incubated at 33, 35, 37, 39, and 41 °C at 150 r/min for 24 h before FPA measurement.

1.5.3 Effect of Shaker Speed on Enzyme Production

Medium initial pH was set at 6.5, 4% inoculum was added to 100 mL medium in 250 mL flasks, and shaker speed was set at 100, 125, 150, 175, and 200 r/min.

Cultures were incubated at 35 °C for 24 h and FPA was measured.

1.5.4 Effect of Liquid Medium Volume on Enzyme Production

Medium volumes of 60, 80, 100, 120, and 140 mL were used in 250 mL flasks. Medium initial pH was 6.5, 4% inoculum was added, and cultures were incubated at 125 r/min, 35 °C for 24 h before FPA measurement.

1.5.5 Orthogonal Optimization Experiment

Based on single-factor experiment results, an orthogonal experiment was designed using four factors: medium initial pH (A), culture temperature (B), shaker speed (C), and liquid medium volume (D). An L9(3) orthogonal array was employed to determine the optimal enzyme production conditions.

2.1.1 Primary Screening

As shown in [Figure 1: see original paper], transparent zones around colonies were clearly visible after iodine staining at 24 h. Two colonies produced transparent zones (indicated by arrows in Figure 1), with the left strain designated LD and the right strain designated DL.

2.1.2 Secondary Screening

2.1.2.1 Enzyme Activity Assay Results

FPA measurement results are presented in . Based on FPA comparison, strain DL was selected as the cellulose-degrading bacterium. The purified strain was preserved on slant medium at 4 °C.

2.1.2.2 Filter Paper Decomposition Test Results

Strain DL was inoculated into medium and observed for 7 days. As shown in [Figure 2: see original paper], flocculent material appeared at the filter paper edges on day 2; increased flocculation and partial filter paper loss occurred on day 3; the fan-shaped filter paper completely disintegrated into debris by day 5 with medium turbidity; by day 7, extensive bacterial growth adhered to flask walls, the filter paper was substantially decomposed, and medium turbidity increased further. These results demonstrate that strain DL isolated from panda feces produces cellulase and possesses cellulose-degrading capability.

2.2.1 Morphological and Physiological-Biochemical Characteristics

As shown in [Figure 3: see original paper], colonies were circular with irregular serrated margins, convex, opaque, milky white, and 1.0–1.5 cm in diameter. According to , strain DL was Gram-positive, aerobic, and positive for spore

staining, catalase, oxidase, and V-P tests, but negative for methyl red, indole, and hydrogen sulfide tests. The strain could utilize glucose, fructose, mannose, lactose, starch, and CMC-Na, preliminarily identifying it as a *Bacillus* species or variant.

2.2.2 16S rDNA Sequencing Analysis

PCR amplification of strain DL' s 16S rDNA yielded a 1,400 bp fragment characteristic of 16S rDNA. BLAST homology analysis of the sequencing results ([Figure 4: see original paper]) showed the highest similarity (99%) with *Paenibacillus cookii* LZ033 (GenBank accession JQ073763). A phylogenetic tree was constructed using MEGA 7.0 ([Figure 5: see original paper]).

2.3.1 Effect of Medium Initial pH on Enzyme Production

Medium pH significantly affects microbial growth by influencing enzyme activity during metabolism and nutrient absorption [26]. As shown in [Figure 6: see original paper], FPA increased with pH from 5.5 to 6.5, reaching a maximum of 88.7 U/mL at pH 6.5. FPA decreased at pH 7.0–7.5, with a minimum of 58.8 U/mL at pH 7.5. Based on these results, pH levels of 6.0, 6.5, and 7.0 were selected for orthogonal testing.

2.3.2 Effect of Culture Temperature on Enzyme Production

Within a certain range, microbial growth and metabolite synthesis depend on temperature elevation; however, excessive temperatures inhibit metabolite synthesis, particularly enzymes. As shown in [Figure 7: see original paper], FPA increased with temperature from 33 to 35 °C, reaching a maximum of 91.1 U/mL at 35 °C. FPA decreased from 37 to 41 °C, with the lowest value at 41 °C, as high temperatures were unsuitable for strain DL growth and enzyme production. Temperature levels of 33, 35, and 37 °C were selected for orthogonal testing.

2.3.3 Effect of Shaker Speed on Enzyme Production

Shaking during culture enhances oxygen contact and increases dissolved oxygen while improving nutrient contact for better utilization. As shown in [Figure 8: see original paper], FPA increased with shaker speed from 100 to 125 r/min, reaching 89.0 U/mL at 125 r/min. FPA decreased at speeds above 125 r/min, possibly due to limited oxygen requirements of strain DL. Shaker speed levels of 100, 125, and 150 r/min were selected for orthogonal testing.

2.3.4 Effect of Liquid Medium Volume on Enzyme Production

Liquid medium volume also affects dissolved oxygen; excessive volume reduces oxygen content and inhibits aerobic bacteria, while insufficient volume is also

detrimental. As shown in [Figure 9: see original paper], FPA increased with volume, reaching a maximum of 84.2 U/mL at 120 mL. However, FPA decreased to 58.7 U/mL at 140 mL, likely because strain DL is aerobic and low oxygen levels inhibited growth and enzyme production. Volume levels of 100, 120, and 140 mL were selected for orthogonal testing.

2.3.5 Orthogonal Optimization Experiment Results

2.3.5.1 Orthogonal Optimization of Enzyme Production Conditions

Orthogonal experiment results are shown in . Range analysis indicated that factors affecting FPA, in descending order, were temperature, shaker speed, liquid medium volume, and medium initial pH. The optimal enzyme production conditions were A1B2C2D1: medium initial pH 6, culture temperature 35 °C, shaker speed 125 r/min, and 100 mL medium volume in 250 mL flasks.

2.3.5.2 Verification of Optimized Conditions

The optimal combination A1B2C2D1 did not appear in the orthogonal array, requiring comparison with the best combination from the array (A1B2C2D2). Results are shown in . The range analysis-derived combination A1B2C2D1 produced slightly higher FPA than the orthogonal array's best combination. Therefore, the optimal enzyme production conditions were determined as: medium initial pH 6, culture temperature 35 °C, shaker speed 125 r/min, and 100 mL medium volume in 250 mL flasks, yielding FPA of 102.3 U/mL—a 1.2-fold increase over pre-optimization values.

The giant panda' s diet consists primarily of tough, difficult-to-digest bamboo, which can damage the digestive tract. Panda feces contain mostly bamboo segments, indicating extremely low bamboo utilization efficiency. Zhu et al. [27] studied the panda gut microbiome metagenome and identified cellulase genes, confirming the presence of cellulose-degrading microorganisms. Ma Hailing [19] cultured panda fecal microorganisms and screened for cellulose-degrading fungi and actinomycetes using Congo red and filter paper collapse tests. Liu Yanhong et al. [28] isolated cellulose-degrading fungi from panda feces. This study used beef extract peptone medium to screen for cellulase-producing bacteria because bacteria grow rapidly with short fermentation times, cellulase-producing bacteria are readily obtainable with high expression levels, and they exhibit good thermal stability suitable for genetic engineering applications.

CMC-Na was used as the sole carbon source, and iodine staining was employed to preliminarily assess cellulose degradation capability. CMC-Na is decomposed by cellulase into cellobiose and glucose; iodine cannot form brown complexes with these sugars but can complex with CMC-Na, resulting in transparent zones only around cellulose-degrading colonies. Li Zhengming [26] used iodine staining to screen cellulase-producing strains from rotten wood and humus soil. Since iodine staining only preliminarily reflects cellulase production, secondary screening was necessary. During secondary screening, cellulase activity was measured

and strains with high activity were subjected to filter paper decomposition tests. As filter paper is primarily cellulose, this test further verified cellulose-degrading capability; growth on filter paper as the sole carbon source with visible decomposition indicated strong cellulase production.

16S rDNA sequence homology analysis is the standard method for bacterial identification, combined with phylogenetic analysis and physiological-biochemical characteristics for accurate classification. Fan Cheng et al. [21], Rong Hua et al. [25], and Yang Weiping et al. [29] all isolated cellulose-degrading bacteria from animal intestines and used physiological-biochemical characterization combined with 16S rDNA analysis for identification.

Culture conditions significantly affect microbial growth and metabolite accumulation. Strains with rapid growth and high enzyme production are typically selected for microecological preparation development, requiring optimization of culture conditions to enhance metabolite synthesis. This study improved cellulase activity by optimizing medium initial pH, culture temperature, shaker speed, and liquid medium volume. Cao Hanwen et al. [5] isolated an aerobic cellulose-degrading *Pseudomonas poae* RE*1-1-14 from panda feces and optimized its enzyme production conditions (optimal pH 6, temperature 26 °C, shaker speed 150 r/min, 30% liquid volume), achieving maximum enzyme activity. The strain isolated in this study,* Paenibacillus cookii* LZ033, exhibited different optimal conditions (pH 6, temperature 35 °C, shaker speed 125 r/min, 100 mL liquid volume in 250 mL flask), demonstrating strain-specific differences despite both originating from panda feces.

The isolated strain *Paenibacillus cookii* LZ033 belongs to the order *Bacillales*. Bacilli are among the most widely used probiotics in microecological preparations. Fast-growing, high enzyme-producing strains can rapidly colonize the intestinal tract and help maintain microecological balance [29]. The *Paenibacillus cookii* LZ033 strain obtained in this study secretes cellulase and shows potential for application in feed additives or panda microecological preparations.

Conclusion

1. The cellulose-degrading strain DL isolated from giant panda Lin Bing' s feces was identified as *Paenibacillus cookii* LZ033 based on morphological observation, physiological-biochemical characteristics, and 16S rDNA sequence homology analysis.
2. The optimal enzyme production conditions for strain DL were: medium initial pH 6, culture temperature 35 °C, shaker speed 125 r/min, and 100 mL medium volume in 250 mL flasks. Under these conditions, FPA reached 102.3 U/mL after 24 h cultivation.

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