Screening of Cellulose-Degrading Bacteria from Giant Panda Feces and Optimization of Enzyme Production Conditions: Postprint

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Abstract

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

Full Text

Screening of Cellulose-Degrading Bacteria from Giant Panda Feces and Optimization of Their Enzyme Production Conditions

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Abstract: This study aimed to screen and identify cellulose-degrading bacterial strains from giant panda feces and optimize their 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 bacterial 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—medium initial pH, culture temperature, shaker speed, and liquid medium volume—were selected. Building upon single-factor experimental results, orthogonal testing was employed to determine the optimal conditions: medium initial pH of 6, culture temperature of 35 °C, shaker speed of 125 r/min, and 100 mL liquid medium in a 250 mL flask. Under these conditions, cellulase activity (expressed as filter paper activity) reached 102.3 U/mL.

Keywords: giant panda feces; cellulose-degrading bacteria; 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 endangered species in 1984. Subadult pandas transition to a high-fiber bamboo-based diet at around 10 months of age, with each adult consuming 12–38 kg of bamboo daily. However, pandas can only utilize 8% of cellulose and 27% of hemicellulose from bamboo. The panda' s digestive system is typical of carnivorous mammals. Analysis of the published giant panda genome sequence in 2010 revealed genes encoding enzymes associated with carnivorous digestive systems but no cellulase genes, indicating that cellulose digestion in pandas relies primarily on gut microorganisms. The giant panda intestine consists only of small and large intestines, is relatively short, and contains high oxygen levels, suggesting it may be more suitable for aerobic or facultatively anaerobic microorganisms. Any alteration in the gut microbiota can cause digestive disorders and even death. Therefore, studying the intestinal microbial flora is crucial for preventing digestive diseases, improving panda health, and developing microecological preparations. While many cellulose-degrading microorganisms exist in nature, the endangered status of pandas necessitates safety considerations. Strains isolated from panda feces are more suitable as microecological preparations or feed additives than those from other environments.

Recent years have seen increased attention to giant panda gut microbes. Previous studies have isolated and identified panda gut flora, screened cellulase-producing *Serratia* and *Bacillus* strains from panda feces, and isolated *Bacillus* strains that both decompose cellulose and inhibit intestinal pathogens. This

study aimed to isolate an aerobic cellulose-degrading bacterium from the feces of a healthy, non-diarrheic giant 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

The experimental sample consisted of fresh feces collected from a healthy, non-diarrheic female giant panda Lin Bing (born in 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
- **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
- **Fermentation medium:** KH_2PO_4 1 g, glucose 6 g, peptone 8 g, $MgSO_4$ 0.5 g, distilled water 1000 mL

1.3.1 Enrichment Culture

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

1.3.2 Primary Screening

The enriched panda fecal microbial mixture was subjected to standard 10-fold serial dilution. Aliquots (100 µL) of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} 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, left to stand for 3 min, and transparent zones around colonies were observed.

1.3.3 Rescreening

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

1.3.3.1 Enzyme Activity Assay Cellulase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method. Total cellulase activity is typically expressed as filter paper activity (FPA). FPA is defined as the amount of enzyme required to produce 1 µmol of glucose per hour from the substrate. FPA (U/mL) is calculated as follows:

FPA (U/mL) = (glucose × enzyme solution volume × 5.56) / (enzyme amount in reaction system × 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, with an uninoculated control. Cultures were incubated at 37 °C with shaking at 150 r/min for 7 days, with daily photographic documentation of filter paper decomposition.

1.4.1 Morphological Observation and Physiological-Biochemical Characteristics

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

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. PCR products were sequenced by Shanghai Sangon Biological Engineering Technology & Services 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. Bacterial suspension was inoculated at 4% (v/v) into 250 mL flasks containing 100 mL medium, then cultured at 37 °C with shaking at 150 r/min for 24 h before measuring FPA.

1.5.2 Effect of Culture Temperature on Enzyme Production

Medium initial pH was adjusted to 6.5, and bacterial suspension was inoculated at 4% into 250 mL flasks containing 100 mL medium. Cultures were incubated at 33, 35, 37, 39, and 41 °C with shaking at 150 r/min for 24 h before measuring FPA.

1.5.3 Effect of Shaker Speed on Enzyme Production

Medium initial pH was adjusted to 6.5, and bacterial suspension was inoculated at 4% into 250 mL flasks containing 100 mL medium. Shaker speeds were set at 100, 125, 150, 175, and 200 r/min, with incubation at 35 °C for 24 h before measuring FPA.

1.5.4 Effect of Liquid Medium Volume on Enzyme Production

Different volumes of medium (60, 80, 100, 120, and 140 mL) were added to 250 mL flasks. Medium initial pH was 6.5, bacterial suspension was inoculated at 4%, and cultures were shaken at 125 r/min at 35 °C for 24 h before measuring FPA.

1.5.5 Orthogonal Optimization Experiment

Based on single-factor 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 $L_9(3^4)$ orthogonal array was employed to determine the optimal enzyme production conditions for the cellulose-degrading bacterium.

2.1.1 Primary Screening

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

2.1.2 Rescreening

2.1.2.1 Enzyme Activity Assay Results

FPA measurement results are presented in . By comparing FPA values, strain DL was selected as the cellulose-degrading bacterium. The purified strain was transferred to preservation medium and stored 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 edges

of intact filter paper on day 2; flocculent material increased and filter paper showed partial loss on day 3; the fan-shaped filter paper completely disintegrated into paper debris and the medium became turbid by day 5; by day 7, wall adhesion was observed with abundant bacterial growth, and the filter paper was essentially decomposed with the medium becoming more turbid. These results demonstrate that strain DL isolated from giant panda feces can produce cellulase and possesses cellulose-degrading capability.

2.2.1 Morphological Observation and Physiological-Biochemical Characteristics

As shown in [Figure 3: see original paper], colonies were circular with irregular serrated edges, convex surfaces, opaque milky color, and diameters of 1.0–1.5 cm. According to , strain DL is 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 can utilize glucose, fructose, mannose, lactose, starch, and CMC-Na, preliminarily identifying it as a *Bacillus* species or variant.

2.2.2 16S rDNA Sequencing Results Analysis

PCR amplification of 16S rDNA from strain DL yielded a 1,400 bp fragment characteristic of 16S rDNA. Sequence analysis ([Figure 4: see original paper]) via BLAST showed that strain DL had the closest phylogenetic relationship with *Paenibacillus cookii* LZ033, with 99% homology and NCBI accession number 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. As shown in [Figure 6: see original paper], FPA increased within the pH range of 5.5–6.5, 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 the orthogonal experiment.

2.3.2 Effect of Culture Temperature on Enzyme Production

Within a certain range, microbial growth and metabolite synthesis depend partially on temperature elevation. However, excessively high temperatures inhibit metabolite synthesis, particularly enzymes. As shown in [Figure 7: see original paper], FPA increased gradually with temperature from 33–35 °C, reaching a maximum of 91.1 U/mL at 35 °C. FPA decreased progressively from 37–41 °C, with the lowest value at 41 °C, indicating that high temperatures were un-

suitable for strain DL growth and enzyme production. Based on these results, temperatures of 33, 35, and 37 °C were selected for the orthogonal experiment.

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 100–125 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. Based on these results, shaker speeds of 100, 125, and 150 r/min were selected for the orthogonal experiment.

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 bacterial growth, while insufficient volume is also detrimental. As shown in [Figure 9: see original paper], FPA increased gradually with medium 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. Based on these results, liquid medium volumes of 100, 120, and 140 mL were selected for the orthogonal experiment.

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 the four factors affected FPA in descending order: 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 liquid medium in a 250 mL flask.

2.3.5.2 Verification Test of Optimized Conditions

The optimal condition combination A1B2C2D1 did not appear in the orthogonal array, requiring comparison with the best combination from the orthogonal table (A1B2C2D2). Results are shown in . The combination A1B2C2D1 from range analysis produced slightly higher FPA than the orthogonal table' s optimal 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 liquid medium in a 250 mL flask, yielding FPA of 102.3 U/mL—a 1.2-fold increase compared to pre-optimization.

The giant panda' s diet consists primarily of tough, difficult-to-digest bamboo, which can easily damage the digestive tract. Panda feces contain mostly bamboo fragments, indicating extremely low bamboo utilization efficiency. Metagenomic studies of panda gut microbiota have identified cellulase genes, confirming the presence of cellulose-degrading microorganisms. Previous research has isolated cellulose-degrading fungi and actinomycetes from panda feces using Congo red and filter paper collapse tests. 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 obtained with high expression levels, and they exhibit good thermal stability suitable for genetic engineering applications.

CMC-Na was used as the sole carbon source in the screening medium. Iodine staining forms transparent zones around cellulose-degrading bacteria because CMC-Na is hydrolyzed into cellobiose and glucose, which do not form brown complexes with iodine, while intact CMC-Na does. Iodine staining only preliminarily reflects cellulase production characteristics, necessitating rescreening through enzyme activity measurement and filter paper decomposition tests. Filter paper, composed primarily of cellulose, provides further verification of cellulose-degrading capability—greater decomposition indicates better 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. Various studies have used this approach to identify cellulose-degrading bacteria from animal intestines.

Culture conditions significantly affect microbial growth and metabolite accumulation. Strains with rapid growth and high enzyme production are typically selected targets, 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. Previous research isolated an aerobic cellulose-degrading *Pseudomonas poae* RE*1-1-14 from panda feces with optimal conditions of pH 6, 26 °C, 150 r/min, and 30% liquid volume. In contrast, this study' s* Paenibacillus cookii* LZ033 showed different optimal conditions: pH 6, 35 °C, 125 r/min, and 100 mL/250 mL flask, demonstrating strain-specific differences despite both originating from panda feces.

The isolated strain *Paenibacillus cookii* LZ033 belongs to the order *Bacillales*. *Bacillus* species are among the most widely used probiotics in microecological preparations. Fast-growing, high enzyme-producing strains can rapidly colonize the intestine and help maintain microecological balance when used as feed additives. This *Paenibacillus cookii* LZ033 strain, capable of secreting cellulase, shows potential for application in feed additives or giant panda microecological preparations.

Conclusions

The cellulose-degrading bacterium 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.

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 liquid medium in a 250 mL flask. 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.