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Abstract

aflatoxin-degrading enzyme; genetic recombination; Bacillus subtilis; fermentation

Full Text

Expression and Application of Aflatoxin Detoxification Enzyme Gene in Bacillus subtilis

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Abstract: This study investigated the expression and application of the aflatoxin detoxification enzyme (ADTZ) gene in *Bacillus subtilis*. An integrative expression vector for ADTZ was constructed and transformed into wild-type *B. subtilis* LN, with protein expression initially detected via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fermentation experiments were conducted by inoculating the recombinant *B. subtilis* into moldy corn to evaluate changes in aflatoxin B1 (AFB1) content and verify the expression efficacy of ADTZ in this host. The results demonstrated successful integration of the ADTZ gene into the *B. subtilis* genome, with the recombinant strain secreting a specific protein band detectable by SDS-PAGE. The AFB1 content in moldy corn fermented with the recombinant strain differed significantly from both uninoculated controls and wild-type *B. subtilis* LN treatments ($P < 0.05$). These findings indicate that ADTZ can be successfully integrated into wild-type *B. subtilis* LN, expressed as an extracellular secreted product with biological activity capable of effectively degrading AFB1.

Keywords: aflatoxin detoxification enzyme; gene recombination; *Bacillus subtilis*; fermentation

Aflatoxins (AF) are a class of chemically similar compounds primarily synthesized as secondary metabolites by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* [1-4]. These substances are highly toxic and exhibit strong teratogenic, carcinogenic, and mutagenic effects [5-6]. Aflatoxin detoxification enzyme (ADTZ) is an active substance that specifically degrades aflatoxins. While microbial enzymatic degradation of aflatoxins has attracted considerable attention in recent years, natural ADTZ is difficult to isolate in large quantities from nature due to low abundance and poor activity [7]. Using molecular biology and genetic engineering techniques to clone and express the ADTZ gene in different hosts can enhance both yield and activity, offering substantial potential for developing applications to mitigate aflatoxin contamination. The ADTZ gene originates from the fungus *Armillariella tabescens*, and its expression product is an intracellular enzyme of this fungus [8] capable of degrading natural aflatoxins. As a complete gene sequence, it can express active specific proteins in both *Escherichia coli* and yeast [9-11]; however, its expression in the animal probiotic *B. subtilis* has not been reported [12]. The *B. subtilis* expression system represents an important prokaryotic expression platform with a complete secretory expression mechanism, widely applied in industrial fermentation and serving as a primary production strain and expression host for various enzyme preparations [13]. This study constructed an integrative expression vector for ADTZ using previously developed *B. subtilis* integration vectors and cloned ADTZ genes, transformed it into the *B. subtilis* genome for integrated expression, and verified its aflatoxin degradation capacity through fermentation experiments, aiming to accumulate experience and data for future development of mycotoxin-degrading enzymes and feed detoxification research.

1.1.1 Plasmids, Strains, and Primers

The biological materials and their characteristics used in this experiment are listed in Table 1 . Primers were synthesized by Shanghai Sangon Biotech Co., Ltd.

Table 1 Biological materials and characteristics used in this experiment

Items	Names	Characteristics
Plasmid	Pm-ADTZ	Constructed in preliminary work, containing ADTZ gene (2,088 bp)
Plasmid	PGEM-Kmpgmt	Constructed in our laboratory, *B. subtilis* integrative expression vector [14]
Plasmid	PTM1M2-ADTZ	Constructed in this study, *B. subtilis* integrative vector containing ADTZ gene
Strain	*E. coli* DH5	Stored in our laboratory, cloning host for *E. coli*
Strain	*B. subtilis* LN	Isolated from *Budorcas taxicolor* feces in Qinling Mountains [15]

Items	Names	Characteristics
Strain	*B. subtilis* ADTZ	Constructed in this study, expresses ADTZ gene, used as fermentation strain
Primer	Forward: 5' -ATGGCCACCACAACTGTC-3' Reverse: 5' -TCACAATCGTCTCTCAATG-3'	

1.1.2 Main Reagents

Prestained protein marker (MP203), bacterial genomic DNA extraction kit (DP302), and 1 kb DNA Ladder (MD111) were purchased from Beijing Tiangen Biotech Co., Ltd.; unstained protein marker (SM0431) and DNA marker (SM0331) were from Thermo Fisher Scientific (China) Co., Ltd.; restriction endonucleases SacII and BamHI were from Promega (USA); lysozyme (L8120) was from Beijing Solarbio Science & Technology Co., Ltd.; aflatoxin B1 (AFB1) enzyme-linked immunosorbent assay (ELISA) kit was from Beijing Hu' an Mycotoxin Biotechnology Co., Ltd.; L-tryptophan (L-Trp), casein hydrolysate, agarose, and other reagents were from Luoyang Boguan Trading Co., Ltd.

1.2 Experimental Methods

1.2.1 Construction of ADTZ Gene Integrative Vector Plasmids PGEM-Kmpgmt and Pm-ADTZ were double-digested with SacII and BamHI, followed by gel electrophoresis and recovery. The fragments were ligated with T4 DNA ligase at 16 °C for 12 h. The ligation product was transformed into *E. coli* DH5 competent cells. Positive clones were selected, cultured in liquid Luria-Bertani (LB) medium, and plasmids were extracted. The recombinant plasmid was identified by SacII and BamHI digestion and sequencing, then designated as PTM1M2-ADTZ.

1.2.2 Preparation of Wild-Type *B. subtilis* LN Competent Cells Competent cells of wild-type *B. subtilis* LN were prepared according to the method described in reference [16].

1.2.3 Construction of Recombinant Strain *B. subtilis* ADTZ Five hundred microliters of wild-type *B. subtilis* LN competent cells were mixed with 10 g of PTM1M2-ADTZ plasmid and incubated at 37 °C with shaking at 200 r/min for 60 min. Five hundred microliters of LB liquid medium was added, and the culture was incubated at 37 °C and 200 r/min for 30 min to allow cell recovery. One hundred microliters of the cell suspension was spread onto LB solid medium and incubated upside down at 37 °C for 14–16 h. Colony morphology was observed, and preliminary detection was performed by Gram staining.

Single colonies of the recombinant strain were selected for scale-up culture, and whole-genome DNA was extracted. PCR was performed using the primers listed above to detect the ADTZ gene. Positive *B. subtilis* strains containing ADTZ were designated as *B. subtilis* ADTZ.

1.2.4 SDS-PAGE Analysis of Recombinant *B. subtilis* ADTZ Culture Recombinant *B. subtilis* ADTZ culture was inoculated into 50 mL LB medium at a 1:50 ratio and incubated at 37 °C and 220 r/min for approximately 18 h, with wild-type *B. subtilis* LN as the control. The culture was centrifuged at low speed for 5 min to collect the supernatant and cell pellet. Five milliliters of lysozyme was added to the pellet, resuspended, and incubated at 37 °C for 30 min, followed by sonication for 10 min. The supernatant and lysed cell samples of *B. subtilis* ADTZ, along with control supernatant and pellet samples, were subjected to SDS-PAGE analysis.

1.2.5 Detoxification Experiment of Recombinant Strain on Moldy Corn Approximately 500 g of corn was moistened to 20% water content and incubated at room temperature (25 °C) until black mold was visible on some embryos. The moldy corn was then dried at 65 °C, ground, and thoroughly mixed. The AFB1 content was measured at 63 g/kg. Nine 50 g samples of the moldy corn were weighed into 500 mL flasks, each containing 450 mL distilled water, and sterilized at 121 °C for 20 min. The nine flasks were randomly divided into three groups: control, experimental group 1, and experimental group 2. Fifty milliliters of sterile LB liquid medium, wild-type *B. subtilis* LN culture (viable count 5.0×10 CFU/mL), or *B. subtilis* ADTZ culture (viable count 4.5×10 CFU/mL) was inoculated into the moldy corn, respectively. Fermentation was conducted at 35 °C with shaking at 200 r/min for 48 h, with three replicates per group. After fermentation, AFB1 content was measured using the aflatoxin ELISA kit on a microplate reader, and results were calculated accordingly.

1.3 Statistical Analysis

Experimental data were analyzed by one-way ANOVA using SPSS 11.5. Data are presented as "mean ± standard error." $P < 0.05$ was considered statistically significant.

2.1 Analysis and Identification of Integrative Vector PTM1M2-ADTZ

The extracted integrative vector PTM1M2-ADTZ was analyzed by double digestion with SacII and BamHI. As shown in [Figure 1: see original paper], two fragments of approximately 2,000 bp were observed, consistent with the size of the ADTZ gene, and another fragment of approximately 6,000 bp corresponded to the vector backbone of PGEM-Kmpgmt.

M1 is 1 kb marker (top to bottom: 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 kb); M2 is DL2000 marker (top to bottom: 2,000, 1,000, 750, 500, 250, 100 bp); lanes 1, 2, and 3 show double digestion identification of PTM1M2-ADTZ.

2.2 Colony Morphology and Gram Staining Identification of Recombinant *B. subtilis* ADTZ

Colonies of the recombinant *B. subtilis* ADTZ exhibited a rough surface with a slightly milky white color, irregular edges, central elevation followed by flattening, and irregular wrinkles—characteristics consistent with *B. subtilis* morphology ([Figure 2: see original paper]-A). Gram staining revealed blue-purple, short rod-shaped cells with spores under 1,600× oil immersion microscopy ([Figure 2: see original paper]-C), matching the morphology of wild-type *B. subtilis* LN ([Figure 2: see original paper]-B).

A: Colony morphology of *B. subtilis* ADTZ; B: Gram staining of *B. subtilis* LN; C: Gram staining of *B. subtilis* ADTZ.

2.3 Detection of ADTZ Integration in Recombinant *B. subtilis* ADTZ

Whole-genome DNA was extracted from recombinant *B. subtilis* ADTZ and used as a template for PCR amplification with ADTZ-specific primers. Agarose gel electrophoresis results are shown in [Figure 3: see original paper]. Lanes 4–6 show the whole-genome DNA of recombinant *B. subtilis* ADTZ, with clear bright bands indicating successful extraction. Lanes 1–3 show PCR amplification products of approximately 2,000 bp, consistent with the ADTZ gene fragment size (2,088 bp), providing preliminary confirmation of ADTZ integration into the recombinant genome. Sequencing of the PCR products revealed complete identity with the ADTZ gene sequence, confirming successful integration into wild-type *B. subtilis* LN and construction of recombinant strain *B. subtilis* ADTZ.

M is 1 kb DNA marker (MD111); lanes 1–3 are PCR amplification products of the target gene from *B. subtilis* ADTZ genome; lanes 4–6 are whole-genome DNA of *B. subtilis* ADTZ.

2.4 Expression of ADTZ Gene in Recombinant *B. subtilis* ADTZ

Recombinant *B. subtilis* ADTZ was cultured in shake flasks, and both culture supernatant and cell pellets were collected for SDS-PAGE analysis alongside wild-type *B. subtilis* LN controls. As shown in [Figure 4: see original paper], a protein band of approximately 70 kDa was detected in the supernatant of *B. subtilis* ADTZ, consistent with the expected size of the ADTZ gene product. This band was absent in the wild-type *B. subtilis* LN culture. No distinct protein bands were observed in the lysed cell pellet samples of *B. subtilis* ADTZ, consistent with the extracellular secretion characteristics of *B. subtilis*. Based on these SDS-PAGE results, ADTZ gene expression in recombinant *B. subtilis* ADTZ was confirmed, with the expressed product secreted into the fermentation broth.

Lanes 1–3: Fermentation supernatant of *B. subtilis* ADTZ; lane 4: Fermentation supernatant of wild-type *B. subtilis* LN; M: Protein marker (top to bottom: 116, 66, 45, 35, 25, 18, 14 kDa); lane 5: Lysed cell pellet sample of *B. subtilis* ADTZ.

2.5 Degradation of AFB1 in Moldy Corn by Recombinant *B. subtilis* ADTZ

After processing the fermentation samples according to the kit protocol and analyzing the microplate reader results with ELISA software, the data were compiled as shown in . Microbial fermentation reduced AFB1 content in all moldy corn samples. Experimental group 1, fermented with recombinant *B. subtilis* ADTZ, showed significantly lower AFB1 content and significantly higher degradation rate compared to the other two groups ($P < 0.05$). Wild-type *B. subtilis* LN also degraded some AFB1, but both AFB1 content and degradation rate differed significantly from the recombinant strain ($P < 0.05$).

Table 2 Degradation of AFB1 in moldy corn by recombinant *B. subtilis* ADTZ

Items	Content of AFB1 (g/kg)	Degradation rate of AFB1 (%)
Control group (moldy corn)	60.75 ± 0.43	–
Experimental group 1 (*B. subtilis* ADTZ fermentation)	19.20 ± 0.32	68.40 ± 0.38
Experimental group 2 (*B. subtilis* LN fermentation)	55.00 ± 0.65	9.47 ± 0.57

In the same column, values with different lowercase letter superscripts indicate significant differences ($P < 0.05$).

3.1 Expression of ADTZ Gene in Different Hosts

The ADTZ gene originates from the fungus *Armillariella tabescens*. Previous studies reported heterologous fusion expression of aflatoxin-detoxifying enzyme in *E. coli* BL21(DE3) cells using the pMAL-c2x vector, with active protein detected [10]. Subsequently, an aflatoxin-oxidase (AFO) with AFB1 conversion function was successfully cloned and expressed [17], further confirming the integrity of the ADTZ gene and enabling its expression in the eukaryotic host

Pichia pastoris [11]. This study achieved expression of ADTZ in the animal probiotic *B. subtilis* through integrative vector construction, offering greater feasibility for animal feed applications.

3.2 Advantages of the *Bacillus subtilis* Expression System

The expression host used in this study, wild-type *B. subtilis* LN [15], was isolated and screened from fresh takin feces in the Qinling Mountains and has been thoroughly characterized as suitable for use as an animal probiotic additive. As a probiotic, wild-type *B. subtilis* LN produces no endotoxins, improves feed quality, regulates animal intestinal microbial balance, and enhances feed palatability [18-19]. *Bacillus subtilis* can secrete heterologous proteins directly into the extracellular medium through its secretory pathway, simplifying the cell disruption, purification, and recovery processes required in *E. coli* expression systems and enabling more direct action. SDS-PAGE analysis revealed a specific protein band in the culture supernatant of recombinant *B. subtilis* ADTZ, consistent with the reported size of ADTZ protein [20], while no such band was observed in wild-type *B. subtilis* LN or cell pellet samples. This confirms extracellular secretory expression, a characteristic that facilitates proper protein folding, effectively reduces inclusion body formation, and simplifies purification procedures [13,21], thereby favoring ADTZ expression. Compared with eukaryotic expression systems, *B. subtilis* offers advantages including simple fermentation conditions, short fermentation cycles, no significant codon bias, and low production costs [22].

3.3 Microorganisms and Enzymes for Aflatoxin Degradation

Numerous studies have investigated microorganisms and enzymes capable of degrading aflatoxins. Doyle et al. [23] found that *Aspergillus parasiticus* mycelia produce a lactoperoxidase after 14 h of growth that degrades AFB1 into AFB2a derivatives and another water-soluble derivative with significantly lower toxicity. Motomura et al. [24] isolated a 90 kDa aflatoxin-degrading enzyme from *Pleurotus ostreatus*, identified as an intracellular oxidase from *Armillariella tabescens* based on fluorescence measurements. Lei et al. [25] studied the detoxification activity, antimicrobial properties, and stress resistance of *B. subtilis*, demonstrating that detoxification-active components existed in the fermentation supernatant, with activity significantly reduced after heat and proteinase K treatment, indicating that the active detoxifying substance is a secreted extracellular enzyme. Subsequent reports described *B. subtilis* fermentation supernatants achieving 70–80% degradation of AFB1 standard solutions [26-28]. This study used naturally moldy corn as the detoxification substrate, which differs substantially from standard solutions in both toxin content and presentation. Both recombinant *B. subtilis* ADTZ and wild-type *B. subtilis* LN exhibited aflatoxin-degrading capacity, but with significant differences, indicating that while *B. subtilis* possesses inherent aflatoxin degradation ability, this capacity varies considerably among strains and can be substantially enhanced through genetic

engineering.

4 Conclusion

The ADTZ gene can be successfully integrated into wild-type *B. subtilis* LN and expressed as an extracellular secreted product with biological activity capable of effectively degrading AFB1.

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