

Article

Recombinant Expression of *Trametes versicolor* Aflatoxin B₁-Degrading Enzyme (TV-AFB₁D) in Engineering *Pichia pastoris* GS115 and Application in AFB₁ Degradation in AFB₁-Contaminated Peanuts

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Abstract: Aflatoxins seriously threaten the health of humans and animals due to their potential carcinogenic properties. Enzymatic degradation approach is an effective and environmentally friendly alternative that involves changing the structure of aflatoxins. In this study, *Trametes versicolor* aflatoxin B₁-degrading enzyme gene (TV-AFB₁D) was integrated into the genome of *Pichia pastoris* GS115 by homologous recombination approach. The recombinant TV-AFB₁D was expressed in engineering *P. pastoris* with a size of approximately 77 kDa under the induction of methanol. The maximum activity of TV-AFB₁D reached 17.5 U/mL after the induction of 0.8% ethanol (*v/v*) for 84 h at 28 °C. The AFB₁ proportion of 75.9% was degraded using AFB₁ standard sample after catalysis for 12 h. In addition, the AFB₁ proportion was 48.5% using AFB₁-contaminated peanuts after the catalysis for 18 h at 34 °C. The recombinant TV-AFB₁D would have good practical application value in AFB₁ degradation in food crops. This study provides an alternative degrading enzyme for the degradation of AFB₁ in aflatoxin-contaminated grain and feed via enzymatic degradation approach.

Keywords: aflatoxin B₁; *Pichia pastoris* GS115; peanuts; *Trametes versicolor*; degradation enzyme

Key Contribution: The recombinant TV-AFB₁D expressed in *Pichia pastoris* GS115 could effectively degrade AFB₁ from AFB₁-contaminated peanuts.

1. Introduction

Aflatoxins are pathogenic fungal toxins produced by *Aspergillus flavus* and *A. parasiticus* [1]. Aflatoxins and their producing bacteria are usually present in food products, feedstuffs, and agricultural raw materials [2]. Aflatoxins are a real threat to human and animal health due to their carcinogenicity and teratogenicity [3]. Among more than 20 kinds of aflatoxin derivatives, AFB₁ possesses the highest toxicity of liver cancer and immune system damage. AFB₁ causes carcinogenesis in the presence of the double bond of AFB₁ furan ring by inhibiting RNA synthesis [4]. The lactone ring of AFB₁ is the main toxicity site. The cleavage of lactone ring reduces the biological activity of AFB₁ with the formation of non-fluorescent compound [5]. In addition, a highly reactive AFB₁-8,9-epoxide capable of reacting with DNA is produced under the activation of cytochrome P450 system [6,7].

Aflatoxins mainly pollute cereals, oils, and their products [8]. As an important agricultural raw material, peanut is directly in touch with *A. flavus* and *A. parasiticus* in the soil [9].

Peanut is highly susceptible to the contamination of aflatoxins. Aflatoxin-contaminated peanuts pollute the environment and cause the serious economic damage [10]. Despite remarkable improvements in the technologies of harvesting and storage processing, the contamination of aflatoxins is still a problem to be solved in the peanut industry [11]. Various physical, chemical, and biological methods have been developed to solve the problem of aflatoxin pollution [12]. The physical approach mainly includes heating treatment [13], UV radiation [14], high temperature [15], and adsorption [16]. The main disadvantages are long treatment time and low degradation rate [17,18]. The chemical methods include oxidant treatment and acid–base method by destroying the structure of aflatoxins [19,20]. Further, both the physical and chemical methods could reduce the quality of nutrition by changing the characteristics of raw materials [21].

Currently, the enzymatic degradation approach has been developed for its high specificity and efficiency of catalysis [22]. Laccase [23], peroxidase [24], and reductase [25] have been proved to be capable of degrading aflatoxins. The edible vegetable oils are vulnerable to aflatoxin pollution in the production process due to the raw materials and processing properties. Aflatoxin B₁ is one of the most common toxic substances in edible vegetable oil. Recently, the quality and safety of edible vegetable oils has attracted great attention of China State Council and the State Food and Drug Administration. The government is concentrating R&D efforts to solve the problem of aflatoxin pollution.

Trametes versicolor is a common polypore mushroom found throughout the world and is also a well-known traditional medicinal mushroom, one that is found growing on tree trunks. We cloned AFB₁-degrading enzyme gene from *Trametes versicolor* (TV-AFB₁D) according to Genbank accession number txid717944 in NCBI. TV-AFB₁D was expressed in engineering *E. coli* BL21(DE3) with TV-AFB₁D activity of 9.3 U/mL (not reported). To further investigate the effectiveness of expression, we integrated TV-AFB₁D into the genome of *P. pastoris* GS115 in this study. In addition, the application of TV-AFB₁D for degradation was also investigated in AFB₁-contaminated peanuts. This study provides a new AFB₁-degrading enzyme for application in enzymatic degradation in the food and feed industry.

2. Results

2.1. TV-AFB₁D Cloning and Evolutionary Tree Analysis

Aflatoxin B₁-degrading enzyme TV-AFB₁D from *T. versicolor* was cloned by using the designed primers with RT-PCR technique (Figure 1). The size of TV-AFB₁D was 2100 bp according to electrophoresis analysis and sequencing confirmation. The evolutionary tree analysis of TV-AFB₁D with AFB₁ degradation enzymes from other species is shown in Figure 2. TV-AFB₁D had closer genetic distance with *Trametes coccinea* than other aflatoxin-degrading enzymes from *Dichomitus squalens*, *Polyporus arcularius*, *Lentinus tigrinus*, and *Laetiporus sulphureus*. All the aflatoxin-degrading enzymes from *Trametes* genus belonging to family Polyporaceae speculated that aflatoxin-degrading enzymes would have common traits according to the result of evolutionary tree analysis.

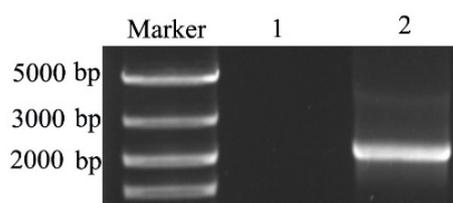


Figure 1. RT-PCR amplification of TV-AFB₁D. Note: lane 1: control; lane 2: TV-AFB₁D.

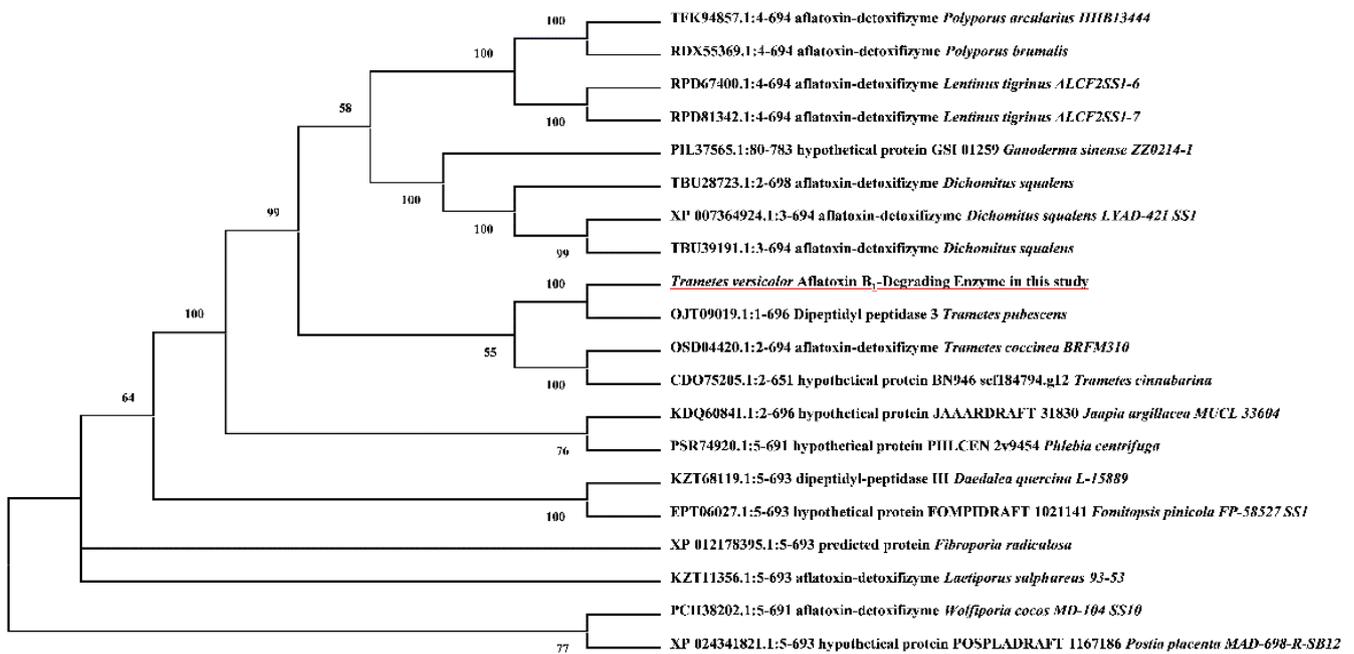


Figure 2. Evolutionary tree analysis of TV-AFB₁D with other AFB₁-degrading enzymes.

2.2. Expression Vector Construction of TV-AFB₁D

TV-AFB₁D was integrated into pPIC9K-His vector by double digestion techniques. The constructed recombinant plasmid was identified by the double digestion of *Sna*B I and *Not* I. Two bands after digestion possessed the size of approximately 9300 and 2100 bp (Figure 3A). The confirmed recombinant vector was named pPIC9K-His-AFB₁D after confirmation by gene sequencing (Figure 3B).

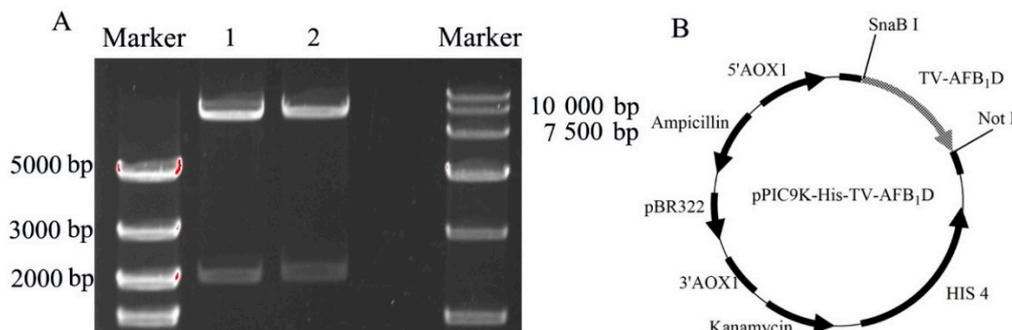


Figure 3. Vector pPIC9K-His-TV-AFB₁D construction. (A) Double-restriction digestion of vector pPIC9K-His-TV-AFB₁D by *Sna*B I and *Not* I. Note: lanes 1, 2: pPIC9k-TV-AFB₁D. (B) Schematic diagram of constructed pPIC9k-TV-AFB₁D.

2.3. Transformation of TV-AFB₁D into *P. pastoris* GS115

The *P. pastoris* transformants were cultured on the plates with MD media after transformation. The transformants were transferred to the plates equipped with MM media (Figure 4). The transformants were identified with the designed primers for TV-AFB₁D amplification. *P. pastoris* GS115 transformants with *His*⁺*Mut*⁺ type for methanol utilization were integrated by pPIC9K-His-AFB₁D according to the growth on the plates containing MD and MM media.

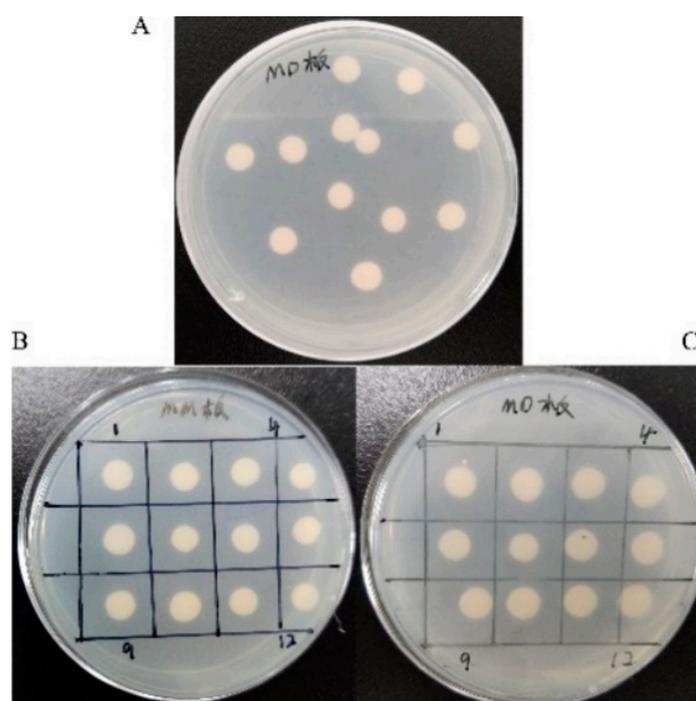


Figure 4. Phenotype screening of recombinant *P. pastoris* GS115. (A) Screening with MD media; (B,C) recombinant *P. pastoris* screening with *His⁺ Mut⁺* phenotype using MM (B) and MD (C) plates.

2.4. Recombinant TV-AFB₁D Expression in *P. pastoris* GS115

The recombinant TV-AFB₁D was expressed by engineering *P. pastoris* GS115 with the size of approximately 77 kDa under the induction of 0.8% methanol. No TV-AFB₁D was detected by the wild-type *P. pastoris* GS115 according to SDS-PAGE analysis (Figure 5). The highest activity of TV-AFB₁D was 17.5 U/mL after the induction of 0.8% methanol (*v/v*) for 84 h at 30 °C. No TV-AFB₁D activity of the fermentation broth from the wild-type *P. pastoris* GS115 was detected under the same treatment conditions (Figure 6). Therefore, the recombinant TV-AFB₁D could express in engineering *P. pastoris* GS115.

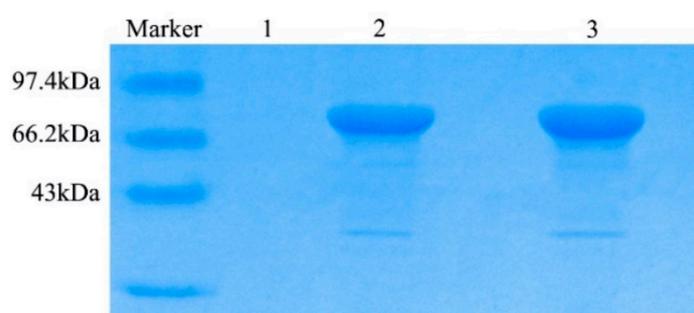


Figure 5. SDS-PAGE analysis of recombinant TV-AFB₁D. Lane 1: wild-type *P. pastoris* expression; lanes 2 and 3: expression of the recombinant TV-AFB₁D.

2.5. Effect of Methanol Concentration and Time on the TV-AFB₁D Expression

The effects of induction time and methanol concentration on TV-AFB₁D activity were investigated (Figure 7). The highest activities of TV-AFB₁D expressed by the recombinant *P. pastoris* GS115 were achieved after induction for 84 h and in the presence of 0.8% methanol (*v/v*). In particular, the activities of TV-AFB₁D were substantially decreased with the addition of 1.2% and 0.5% of methanol. Therefore, the optimum methanol concentration and treatment time were 0.8% and 84 h for the inducible expression of TV-AFB₁D, respectively.

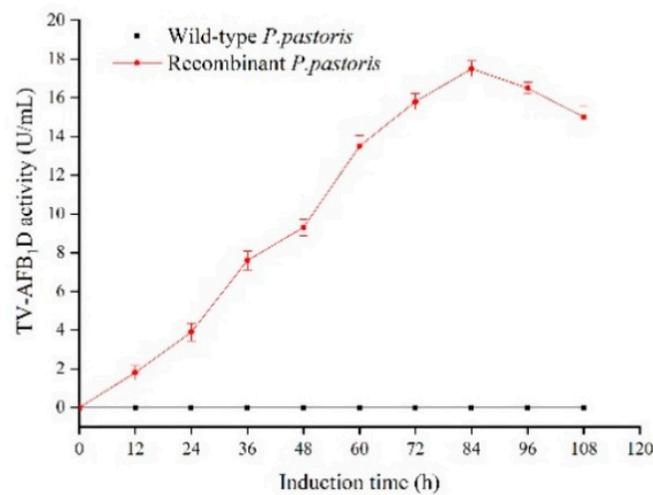


Figure 6. Expression of TV-AFB₁D from the transformants and wild-type *P. pastoris*.

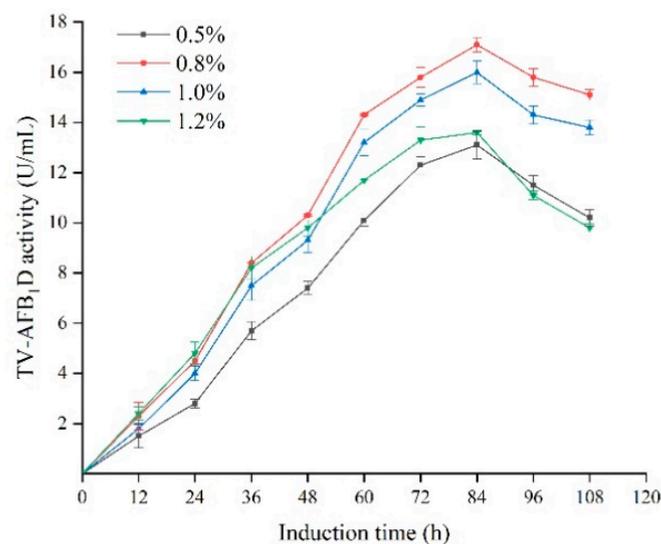


Figure 7. Effect of time and methanol concentration on the activity of TV-AFB₁D.

2.6. Effect of Temperatures and Treatment Time on the Expression of TV-AFB₁D

The effects of temperatures and treatment time on TV-AFB₁D expression are shown in Figure 8. The TV-AFB₁D activities reached the highest after induction for 84 h. The highest activity of TV-AFB₁D was 16.5 U/mL at 28 °C among all the set temperatures of 25–32 °C. Therefore, the optimum temperature and time were 28 °C and 84 h for the inducible expression of TV-AFB₁D in engineering *P. pastoris* GS115, respectively.

2.7. Product Formation of AFB₁-Contaminated Peanuts Catalyzed by TV-AFB₁D

HPLC method was used to determine the product formation of AFB₁ catalyzed by the recombinant TV-AFB₁D. As the control group, the AFB₁ standard possessed a special peak after running for 45 min at a wavelength of 365 nm (Figure 9). The profiles of catalytic products were determined by HPLC after the catalysis of AFB₁-contaminated peanuts by the recombinant TV-AFB₁D. With the exception of the AFB₁ standard, another peak was detected under a wavelength of 210 nm after running for 22 min. The result indicated that new product was formed under the catalysis of TV-AFB₁D with AFB₁ as substrate.

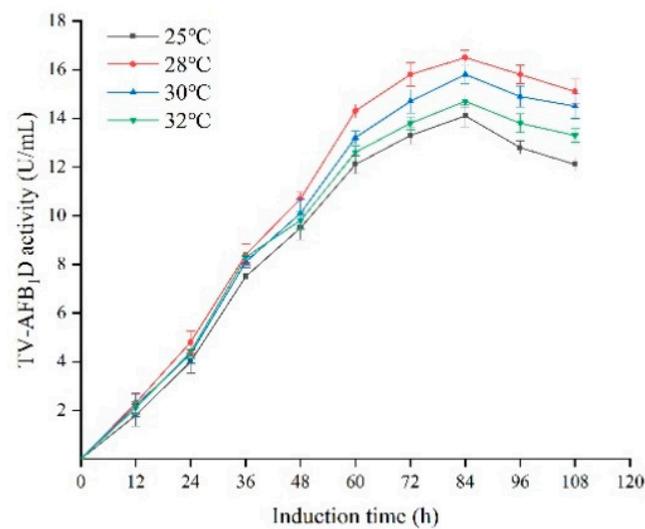


Figure 8. Effect of temperature on the activity of TV-AFB₁D.

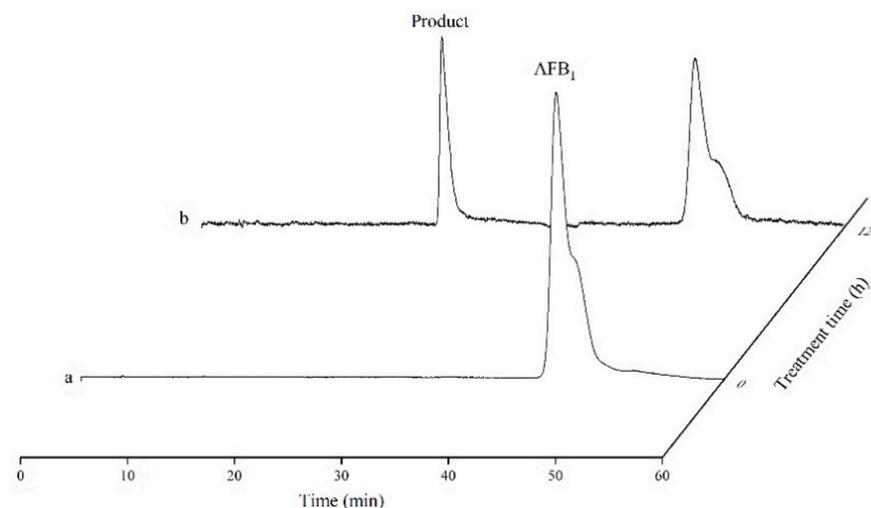


Figure 9. Determination of catalytic products of AFB₁ catalyzed by TV-AFB₁D using HPLC approach. Note: a and b represent HPLC profiles before and after catalysis, respectively.

2.8. Effect of Time on the Residue of AFB₁ Standard Sample

The effect of catalysis time on the residual proportion of the AFB₁ standard sample was investigated at 32 °C (Figure 10). The proportion of AFB₁ residue substantially decreased from 100% to 27.8% for 5 h of catalysis with the degradation rate of 72.2%. During the next catalysis for 5–12 h, the proportion of residual AFB₁ decreased slowly. The proportion of residual AFB₁ standard sample was 24.1% after catalysis for 12 h with the degradation rate of 75.9%.

2.9. Effect of Time on the Proportion of AFB₁ Residue in Peanuts

The effect of treatment time on the proportion of AFB₁ residue in AFB₁-contaminated peanuts was investigated at 32 °C (Figure 11). Within the initial 10 h, the proportion of AFB₁ residue decreased rapidly from 100% to 55.7%. After treatment for 18 h, the proportion of AFB₁ residue in AFB₁-contaminated peanuts gradually decreased to 51.5% with the degradation rate of 48.5%. Therefore, the recombinant TV-AFB₁D could effectively catalyze AFB₁ in AFB₁-contaminated peanuts.

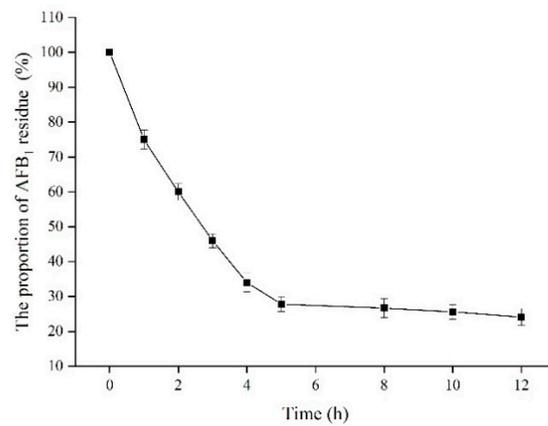


Figure 10. Effect of time on the concentration of AFB₁ standard sample by TV-AFB₁D.

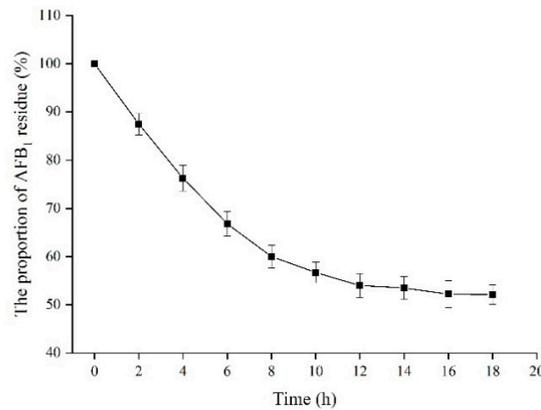


Figure 11. Effect of time on the concentration of AFB₁ in AFB₁-contaminated peanuts by TV-AFB₁D.

2.10. Effect of Temperature on the Proportion of AFB₁ Residue in Peanuts

The effect of temperature on the proportion of AFB₁ residue in AFB₁-contaminated peanuts was investigated after catalysis for 5 h (Figure 12). In the range of 26 to 42 °C, the proportion curve of AFB₁ residue was in the shape of a “V”. The proportion of AFB₁ residue gradually decreased during the temperatures of 26 to 34 °C and increased during the temperatures of 34 to 42 °C. The lowest concentration of AFB₁ residue in AFB₁-contaminated peanuts was 52.1% at 34 °C with a degradation rate of 47.9%. Too low and too high temperatures were not conducive to the degradation of AFB₁ by TV-AFB₁D in AFB₁-contaminated peanuts.

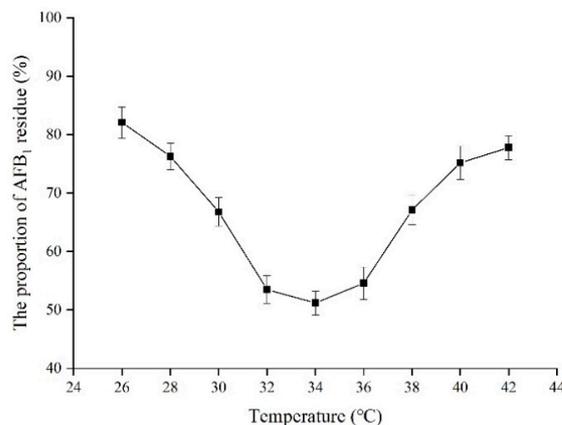


Figure 12. Effect of temperature on the concentration of AFB₁ in AFB₁-contaminated peanuts.

3. Discussion

Although the physical and chemical methods have been applied in the detoxification of aflatoxins, these approaches are difficult to meet the requirement of clean, safe, and environmentally friendly production [26]. Currently, the enzymatic degradation has been applied in the degradation of aflatoxins for its safety and specificity [27]. A large number of studies have been reported on the enzymes related to aflatoxin degradation (Table 1). Enzymes related to aflatoxin degradation include various catalytic properties of F₄₂₀H₂-dependent reductase [25], laccase [23], and peroxidase [24]. In addition, aflatoxin-detoxifzyme (ADTZ) [28], AFB₁-horse radish peroxidase (HRP) [29], *Myxobacteria* aflatoxin degradation enzyme (MADE) [30], *Bacillus* aflatoxin-degrading enzyme (BADE) [31], and *Pantoea* aflatoxin degradation enzyme (PADE) [32] also possess the capability of aflatoxin degradation. In the previous report, the catalytic performances of enzymes were identified by using AFB₁ standard sample as catalytic substrate. These enzymes were rarely used for degradation of aflatoxins in food and feed. In this study, *T. versicolor* aflatoxin B₁-degrading enzyme gene was expressed in engineering *P. pastoris*. AFB₁ standard sample and AFB₁-contaminated peanuts were used to detect the degradation effect of AFB₁. The AFB₁ proportions of 75.9% and 48.5% were degraded using AFB₁ standard sample and AFB₁-contaminated peanuts as substrates, respectively.

Table 1. Aflatoxin-degrading enzymes and catalysis performances.

Microbial Sources and Enzymes	Conditions	Catalytic Efficiency and Time
<i>Mycobacterium smegmatis</i> , F ₄₂₀ H ₂ -dependent reductase [25]	20 mM Tris-HCl, pH 7.5	63%, 4 h
<i>Myxococcus fulvus</i> , MADE [30]	pH 6	96%
<i>Armillariella tabescens</i> , ADTZ [28]	0.02 M PBS, pH 6	80%
<i>Phanerochaete sordida</i> , peroxidase [33]	50 mM PBS, pH 6	86%, 48 h
<i>Pleurotus ostreatus</i> , peroxidase [34]	pH 4.5	90%, 48 h
<i>Bacillus subtilis</i> , BsCotA laccase [35]	50 mM Tris-HCl, pH 7.0	98%, 10 h
<i>Pleurotus pulmonarius</i> , laccase [36]	1 mM sodium acetate, pH 5	90%, 72 h
<i>Aspergillus flavus</i> , HRP [29]	1mM sodium acetate, pH 5	42%, 1 h
<i>Bacillus shackletonii</i> , BADE [31]	50 mM PBS, pH 7.0	47.5%, 72 h
<i>Pseudomonas aeruginosa</i> , PADE [32]	0.02 M PBS, pH 7	72.5%, 12h
<i>T. versicolor</i> , TV-AFB ₁ D, in this study	AFB ₁ standard sample	75.9%, 12 h
<i>T. versicolor</i> , TV-AFB ₁ D, in this study	AFB ₁ -contaminated peanuts	48.5%, 18 h

4. Conclusions

In this study, the recombinant TV-AFB₁D with a size of 77 kDa was effectively expressed by engineering *P. pastoris*. The optimal temperature and methanol concentration were 28 °C and 0.8% for the inducible expression of TV-AFB₁D, respectively. The proportion of residual AFB₁ standard sample was 24.1% after catalysis for 12 h with a degradation rate of 75.9%. In addition, the proportion of AFB₁ residue in AFB₁-contaminated peanuts decreased to 51.5% with a degradation rate of 48.5% after treatment for 18 h at 34 °C. This study provided an alternative TV-AFB₁D degradation enzyme for the removal of AFB₁ in food products and agricultural raw materials.

5. Materials and Methods

5.1. Materials and Reagents

Vector pPIC9K-His, TV-AFB₁D gene, *P. pastoris* GS115, and *T. versicolor* were stored in the Experimental Center of Hefei University of Technology. Gene sequencing and primer synthesis were performed by Sangon Biotech. Taq polymerase, *Stu* I, *Not* I, and *Sna*B I were from NEB Company. Gel imaging systems, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), polymerase chain reaction (PCR) amplification, and electrophoresis devices were manufactured by Bio-RAD Company. ProteinIsoNi-IDA resin and AFB₁ immunoaffinity column were from Transgen Biotech and Welch Company, respectively.

5.2. Construction of pPIC9K-His-TV-AFB₁D Vector

TV-AFB₁D encoding aflatoxin degradation enzyme was cloned by reverse transcription PCR (RT-PCR) technique according to Genbank accession number txid717944 in the National Center for Biotechnology Information (NCBI). The sequences of restriction endonuclease *Sna*B I and *Not* I were added in the corresponding primers for the amplification of TV-AFB₁D. The corresponding primers were designed as follows: F: 5'-GGGAAATACGTAATGGCTCGCGCGAAGTACTC-3' (*Sna*B I); R: 5'-GGGAAAGCGGCCCGCTTAAAGCTTCCGCTCTATGA3' (*Not* I). Both the product of amplification and pPIC9K-His vector were double digested by *Sna*B I and *Not* I. The recovered gene was ligated by T₄ ligase to produce vector pPIC9K-His-AFB₁D. pPIC9K-His-AFB₁D was confirmed by PCR amplification, double-enzyme digestion identification, and gene sequencing.

5.3. Transformation of pPIC9K-His-TV-AFB₁D into *P. pastoris* GS115

The linearized pPIC9K-His-AFB₁D was digested by *Stu* I and integrated into *P. pastoris* GS115 by electroporation transformation method [37]. *P. pastoris* GS115 had a mutation in histidine dehydrogenase (*his4*) and could not synthesize histidine. pPIC-9K-His contained *his4* gene that was complementary to the host. The transformants were screened on the plate equipped with histidine-free medium. The recombinant *P. pastoris* with *His*⁺*Mut*⁺ phenotype grew on minimal methanol medium (MM) plates in the presence of methanol. The transformants were screened out using the solid plates containing minimal dextrose medium (MD) and MM. The true transformants were further confirmed by PCR identification and gene sequencing.

5.4. Inducible Expression and Condition Optimizations of TV-AFB₁D

The *His*⁺*Mut*⁺ phenotype-positive clones were incubated with the shaking speed of 200 rpm at 30 °C. The collected cells of recombinant *P. pastoris* were mixed with methanol-complex buffer medium. The transformants were incubated at 30 °C with the shaking speed of 200 rpm. The fermentation broth was added with 0.8% methanol (*v/v*) every 24 h for the induction of recombinant TV-AFB₁D. The parameters of temperatures, methanol concentrations, and time were optimized to enhance the inducible expression of TV-AFB₁D.

5.5. Purification of Recombinant TV-AFB₁D and Definition of Activity

The recombinant TV-AFB₁D was purified by pre-equilibration with Ni²⁺-chelate affinity chromatography column and elution with imidazole eluent with ProteinIso Ni-IDA resin. The profile of TV-AFB₁D protein was analyzed by SDS-PAGE. The unit of TV-AFB₁D activity was defined as the amount of enzyme that catalyzed the consumption of 1 nmol AFB₁ per minute. The activity of TV-AFB₁D was calculated by using the formula of $(M_0 - M_1)/(312 \times t \times V)$, where M_0 , M_1 , t , and V represent the AFB₁ weight in the control and experimental groups (μ g), reaction time (min), and enzyme amount (mL), respectively; 312 indicates the molecular weight of AFB₁.

5.6. Measurement of AFB₁ Content and Degradation Products

The content of AFB₁ was detected by HPLC using the following process parameters: methanol-to-water ratio of 40/60, isocratic elution, ultraviolet (UV) detector, C₁₈ Shim-pack VP-ODS (5 μ m, 250 mm \times 4.6 mm), C₁₈ reverse column, 0.7 mL/min of flow rate, 190–400 nm of detection wavelength, and 30 °C of column temperature. The signal of AFB₁ was detected by the UV detector at a wavelength of 365 nm. The amount of AFB₁ was calculated on the basis of the peak areas with the AFB₁ standard sample as the control.

5.7. Application in the AFB₁ Degradation of AFB₁-Contaminated Peanuts

The AFB₁-contaminated peanuts were immersed in the solution containing the recombinant TV-AFB₁D from engineering *P. pastoris* GS115. After incubation for 12 h, the recovered peanuts were soaked with methanol solution containing 40% of water (*v/v*). Then, the supernatant was purified through the AFB₁ immunoaffinity column with the

flow rate of 2 drops per second. The collected eluate was further purified by the filtration with 0.22 µm size of filter membrane. The effects of treatment time and temperature on the degradation efficiency of AFB₁-contaminated peanuts by TV-AFB₁D were investigated.

5.8. Data Analysis

The data were statistically given with three replicates. Origin and SPSS software were used to analyze the data with mean ± standard deviation.

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Conflicts of Interest: The authors declare no conflict of interest.

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