Food-Grade Expression of Manganese Peroxidases in Recombinant Kluyveromyces lactis and Degradation of Aflatoxin B₁ Using Fermentation Supernatants

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Aflatoxins are naturally occurring high-toxic secondary metabolites, which cause worldwide environmental contaminations and wastes of food and feed resources and severely threaten human health. Thus, the highly efficient methods and technologies for detoxification of aflatoxins are urgently needed in a long term. In this work, we report the construction of recombinant Kluyveromyces lactis strains GG799(pKLAC1-Phs₃mnp), GG799(pKLAC1-Plo₃mnp), GG799(pKLAC1-Phc₃mnp), and then the food-grade expression of the three manganese peroxidases in these strains, followed by the degradation of aflatoxin B₁ (AFB₁) using the fermentation supernatants. The expression of the manganese peroxidases was achieved in a food-grade manner since Kluyveromyces lactis is food-safe and suitable for application in food or feed industries. The inducible expression process of the optimal recombinant strain GG799(pKLAC1-Phc₃mnp) and the aflatoxin B₁ degradation process were both optimized in detail. After optimization, the degradation ratio reached 75.71%, which was an increase of 49.86% compared to the unoptimized results. The degradation product was analyzed and determined to be AFB₁-8,9-dihydrodiol. The recombinant strain GG799(pKLAC1-Phc₃mnp) supernatants degraded more than 90% of AFB₁ in the peanut samples after twice treatments. The structural computational analysis for further mutagenesis of the enzyme PhcMnp was also conducted in this work. The food-grade recombinant yeast strain and the enzyme PhcMnp have potential to be applied in food or feed industries.

Keywords: mycotoxins, aflatoxin B₁, degradation, Kluyveromyces lactis, food-grade

INTRODUCTION

Aflatoxins are naturally occurring highly toxic secondary metabolites, mainly produced by several species of fungus genus Aspergillus such as A. flavus, A. parasiticus, and A. nomius (Ismail et al., 2018; von Hertwig et al., 2020). Together with other mycotoxins, aflatoxins had caused worldwide contaminations and wastes of food supplies and severely threatened human health...
(Pitt and Miller, 2017; Eskola et al., 2020). There are more than 20 types of structurally similar but different molecules of aflatoxins, in which aflatoxin B$_1$ is the most prominent and dangerous type (Abrar et al., 2013; Loi et al., 2016). In 2012, AFB$_1$ was classified as the Group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (Haque et al., 2020). Further studies and conclusions by IARC and World Cancer Research Fund (WCRF) strongly supported the linkage of AFB$_1$ to liver cancer and other types of cancer risks (Marques et al., 2019; Claeys et al., 2020). In China, the risks of exposure to aflatoxin B$_1$ in foodstuffs or feeds still needed long-term supervision and control (Zhang et al., 2020). Thus, the highly efficient methods and techniques for detoxification of aflatoxin are urgently needed in a long term, to continuously minimize the loss of foodstuffs worldwide and reduce the harmful effects to human beings.

Currently, there are mainly three types of mycotoxin detoxification methods and techniques: physical, chemical, and biological. The physical methods are mainly solvent extraction and absorption, high-temperature degradation, and radiation processing (Di Gregorio et al., 2014; Ismail et al., 2018), and the chemical methods include structural degradation of toxins by some organic reagents such as aldehydes, oxidizing agents (Ismail et al., 2018), and ozone (Wang et al., 2016a,b). The biological methods usually utilize the alive microorganisms (Styriak et al., 2001; Wang et al., 2018), the expressed enzymes (Cao et al., 2011; Wang et al., 2019), and the microbial metabolites (Ghavzini et al., 2016) for structural degradation of toxins. In comparison with the biological methods, the physical and chemical methods have some defects such as the loss of nutrition and the residue of absorbent and chemical compounds, so that the safety and negative effects of using these methods should be continuously investigated (Peng et al., 2018). The biological methods degrade the mycotoxins with mild parameters and in more environment-friendly ways (Loi et al., 2017). However, the direct use of microorganisms still has limitations. For example, many foodstuffs are not suitable for inoculation and proliferation of microorganisms (Assaf et al., 2019), or else the nutrition contents and the texture characteristics might be changed (Peng et al., 2018). Therefore, utilization of extracellular enzymes and culture supernatants might be the best strategies for biological detoxification of mycotoxins (Cao et al., 2011; Feltrin et al., 2017; Zhao et al., 2020).

In this work, we report a new and safe microbial approach for degradation of aflatoxin B$_1$ (AFB$_1$) by the culture supernatants of three food-grade recombinant yeasts, which contained inducibly expressed manganese peroxidases. Manganese peroxidase was originally used in industrial and agricultural applications such as enzymatic bleaching of pulp, treatment of agricultural waste, and treatment of dye wastewater (Ha et al., 2001); in this work, we achieved food-grade expression of the enzymes in yeast and applied the enzyme for the degradation of AFB$_1$ in foodstuff peanuts. The coding genes for manganese peroxidases PhsMnp, PloMnp, and PhcMnp, which originated from *Phanerochaete sordida*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium*, were respectively cloned to expression vector pKLAC1 and then expressed in the food-grade expression host *Kluyveromyces lactis* GG799, which is widely regarded as a food-safe yeast strain and is a suitable organism for the production of food enzymes (van den Dungen et al., 2021). The recombinant strain GG799(pKLAC1-PhcMnp) with the highest AFB$_1$ degradation efficiency was further studied, and the AFB$_1$ degradation parameters were optimized. Besides, the structure of the manganese peroxidase PhcMnp was homology-modeling analyzed for further mutagenesis of this enzyme.

**MATERIALS AND METHODS**

**Chemicals, Strains, and Culture Techniques**

AFB$_1$ was purchased from Pribolab (Qingdao, China), which was made into a 1.0-mg/ml stock solution with methanol or acetonitrile solvent and was preserved and refrigerated in the darkness at $−20^\circ$C. DNA marker, restriction enzymes (*Bgl*II, *Sal*I, *Sac*II, etc.), T4 DNA ligase, and plasmid extraction kits were purchased from Thermo Fisher Scientific (Shanghai, China). Sorbitol and hemin were purchased from Aladdin (Shanghai, China). Fungus genomic DNA extraction kit and growth media components were purchased from Sangon Biotech (Shanghai, China). Methanol, acetonitrile, and formic acid were purchased from Tedia (Fairfield, OH, United States). All other reagents and chemicals were of analytical reagent grade. The genome DNA of *K. lactis* recombinants was extracted and purified by the fungus genomic DNA extraction kit. All the DNA sequencing works were done in Sangon Biotech (Shanghai, China).

For cultivation of *K. lactis* GG799 and the recombinant strains GG799(pKLAC1-PhsMnp), GG799(pKLAC1-PloMnp), and GG799(pKLAC1-PhcMnp), the strains were grown in YEPD liquid (1.0% yeast extract, 2.0% peptone, 2.0% glucose, and pH 6.3) at 30$^\circ$C and 200 rpm for 18 h. For the inducible expression of manganese peroxidases in the recombinant strains, the strains were cultivated in YEPG medium (1.0% yeast extract, 2.0% peptone, 2.0% galactose, 1.0 mmol/l MnSO$_4$, 1.0 mmol/l hemin, pH 6.5) under aerobic conditions at 30$^\circ$C and 200 rpm for 96 h.

**Construction of Recombinant Plasmids and Expression Strains**

Three manganese peroxidases known to be capable of degrading AFB$_1$ in the original strains were selected. Two manganese peroxidase genes from *P. sordida* (Wang et al., 2011) and *P. ostreatus* (Yehia, 2014) were synthesized and named PhsMnp and Plomnp, respectively, and the manganese peroxidase gene from *P. chrysosporium* (Gowda et al., 2007) was constructed and named PhcMnp by adding a glutathione-S-transferase (GST) tag to its 5’-end, for the GST tag can be co-expressed with proteins to increase the solubility of easily aggregated proteins (Tarazi et al., 2021). The three synthesized genes were inserted in the pKLAC1 vector to the *Bgl*II and *Sal*I sites and then linked by T4 DNA ligase to obtain the constructs (pKLAC1-PhsMnp, pKLAC1-PloMnp, and pKLAC1-PhcMnp). These plasmids were transformed into the host strain *E. coli* DH5$\alpha$ for screening of positive clones by PCR verification.
FIGURE 1 | Flowchart for constructions of recombinant plasmids and strains. (A) Flowchart for constructions of recombinant plasmids. (B) The integration mechanism for construction of recombinants using K. lactis GG799(pKLAC1-Phc
mnp) as an example.

(detailed PCR program shown in Supplementary Table 1), restriction digestion of the plasmids, and DNA sequencing. The verified recombinant plasmids were amplified in the E. coli hosts and then transformed into the yeast host K. lactis GG799, for the construction of recombinant yeast strains. Detailed construction procedures for recombinant plasmids and yeast strains are shown in Figure 1. The result recombinant yeast strains were named GG799(pKLAC1-Phs
mnp), GG799(pKLAC1-Plo
mnp), and GG799(pKLAC1-Phc
mnp), respectively.

Inducible Expression Methods of the Enzymes
The recombinant strains K. lactis GG799(pKLAC1-Phs
mnp), GG799(pKLAC1-Plo
mnp), and GG799(pKLAC1-Phc
mnp) were cultivated in YEPD liquid. When OD₆₀₀ reached 1.0, 1% of the cultures were transferred into the YEPG liquid for the inducible expression of the manganese peroxidases, according to the methods described above. The supernatants of these cultures were collected by centrifugation and concentrated by 10-kDa ultrafiltration for subsequent experiments.

Degradation System of AFB₁ by the Enzymes
The AFB₁ degradation process was conducted with the supernatants obtained from the recombinant strains GG799(pKLAC1-Phs
mnp), GG799(pKLAC1-Plo
mnp), and GG799(pKLAC1-Phc
mnp). The total volume of the reaction system was 1.0 ml, containing 50.0 mmol/l malonic acid buffer (pH 4.5), 2.0 µg/ml AFB₁, 1.0 mmol/l MnSO₄, 2.0 mmol/l glucose, 1.0 U/ml glucose oxidase, and 1.0 mg/ml culture supernatant protein, while the protein was replaced with 50.0 mmol/l malonic acid buffer (pH 4.5) in blank control. The solution containing all the samples needed was incubated at 40°C for 40 h, followed by enzyme inactivation in a water bath at
80°C for 10 min. The samples were filtered through the 0.22-µm membranes for removal of the impurities and then subjected to UPLC-TQD analysis.

Quantitative Assay of AFB$_1$

The UPLC-TQD assay was used for qualitative and quantitative analyses of the concentration of AFB$_1$ in samples. The specific operating parameters were as follows, UPLC chromatographic conditions: column: C18; flow rate: 0.30 ml/min; column temperature: 40°C; mobile phases: H$_2$O (buffer A) and acetonitrile (buffer B). MS (Mass) spectrometry conditions: ion source: electrospray ion source; MS spectrometry scanning mode: multiple reaction monitoring mode (MRM); cone hole voltage: 3.0 kV; heating gas temperature: 500°C; ion source temperature: 150°C; desolvation gas: 800 l/h.

The AFB$_1$ standard samples were prepared in acetonitrile with the concentration gradients (100.0, 200.0, 500.0, 1,000.0, 2,000.0, and 5,000.0 ng/ml) to establish the standard curve for AFB$_1$ concentration calculation.

Optimization of Inducible Expression Conditions for GG799(pKLAC1-Phcmnp)

The recombinant strain _K. lactis_ GG799(pKLAC1-Phcmnp) was subjected to different induction conditions (temperature: 15–35°C, time: 24–120 h, rotation speed: 0–300 rpm, hemin concentration: 0.1–5.0 mmol/l, initial pH of induction medium: 3.0–9.0, MnSO$_4$ concentration: 0.1–5.0 mmol/l, galactose concentration: 5.0–80.0 g/l). The enzyme was expressed and secreted to the supernatants as reported (Gnanamani et al., 2006). The expression levels of enzyme PhcMnp were optimized with these parameters univariately and characterized by the degradation ratio of AFB$_1$.

On the basis of the single-factor test, seven factors (temperature, time, rotation speed, hemin concentration, initial pH of induction medium, MnSO$_4$ concentration, and galactose concentration) with different levels were designed for the orthogonal test. The test factors and levels are shown in Supplementary Table 2.

Optimization of Degradation Parameters for AFB$_1$ Degradation by Supernatants of GG799(pKLAC1-Phcmnp)

Under the optimal induced expression conditions, the degradation reaction conditions for AFB$_1$ by the supernatants of recombinant strain GG799(pKLAC1-Phcmnp) were further optimized by setting the concentration gradient, according to the methods reported previously (Wang et al., 2011). For the optimization of single factors by testing the degradation ratio under different reaction conditions, reaction time (4–48 h), temperature (20–60°C), pH (3.0–5.5), protein concentration (0.4–8.0 g/l), MnSO$_4$ concentration (0.2–10.0 mmol/l), glucose concentration (1.0–3.5 mmol/l), and glucose oxidase concentration (0.2–5.0 U/ml) were investigated.

Seven factors (time, temperature, pH, protein concentration, MnSO$_4$ concentration, glucose concentration, and glucose oxidase concentration) and levels of the orthogonal test were also designed (Supplementary Table 3).

Degradation Methods of AFB$_1$ in Peanuts by the Enzymes

According to the methods reported previously (Yang et al., 2020), the peanut samples were shelled, the impurities and dust of which were removed. The samples were ground into powders by homogeneity, with the particle size less than 2.0 mm. The powder samples (5.0 g) were added with 10.0 ml water, stirred thoroughly, followed by sterilization in an autoclave.

The AFB$_1$ standards were added to the peanut samples at different concentrations (50.0, 500.0, and 2,000.0 µg/kg), and the samples were stirred thoroughly. For AFB$_1$ degradation tests, the samples were mixed with 1.2 mmol/l MnSO$_4$, 2.5 mmol/l glucose, 1.5 U/ml glucose oxidase, and the supernatants (containing proteins concentration: 3.0 g/l) for enzymatic degradation reactions. The final volume of the reaction system was 35.0 ml. The reaction was carried out at 40°C, pH of 4.5, for 40 h. All the samples were analyzed by HPLC-MS spectrum (UPLC-TQD) assay to detect the residual concentration of AFB$_1$ in the samples.

RESULTS

Construction of Recombinant Strains _Kluyveromyces lactis_ GG799(pKLAC1-Phs$mnp$), GG799(pKLAC1-Plo$mnp$), and GG799(pKLAC1-Phc$mnp$).

Three recombinant plasmids pKLAC1-Phs$mnp$, pKLAC1-Plo$mnp$, and pKLAC1-Phc$mnp$ were constructed in this work according to the methods described above. The flowchart for the construction is shown in Figure 1A. The vector pKLAC1 and genes Phs$mnp$, Plom$np$, and Phcm$np$ were 9,091, 1,164, 1,116, and 1,824 bp (with a GST tag fused on the 5′-terminal), respectively. Plasmid verification results using agarose gel electrophoresis are shown in Supplementary Figure 1, and the plasmids with correct digestion results were subjected to DNA sequencing verification.

The recombinant plasmids were transferred _K. lactis_ GG799 host, and the transformants were selected and verified by PCR amplification, as shown in Supplementary Figure 2, which indicated successful construction of the recombinant strains of _K. lactis_ GG799(pKLAC1-Phs$mnp$), GG799(pKLAC1-Plo$mnp$), and GG799(pKLAC1-Phc$mnp$).

Inducible Expression of Enzymes and Degradation of AFB$_1$ by the Culture Supernatants of the Recombinant Strains

To express inducibly for manganese peroxidases, the recombinant strains GG799(pKLAC1-Phs$mnp$), GG799(pKLAC1-Plo$mnp$), and GG799(pKLAC1-Phc$mnp$) were firstly cultivated in YEPD liquid for accumulation of the biomass and then induced in YEPG liquid.

The activities of manganese peroxidases were characterized by AFB$_1$ degradation ratios. The supernatants of the recombinant...
strains were used for AFB1 degradation, and the remaining contents of AFB1 were analyzed by HPLC-TQD. A comparison of the liquid chromatogram of AFB1 degradation results by the three recombinants’ supernatants is shown in Figure 2. It can be seen that the peak area of degradation residue by PhcMnp was the smallest in the three samples, which indicated that the enzyme PhcMnp has the best enzyme activity. The degradation ratios of AFB1 by the three recombinants’ supernatants are shown in Table 1. As shown in this table, the supernatant of recombinant strain GG799(pKLAC1-Phc\textsubscript{mnp}) had the highest degradation ratio for AFB1 (50.52 ± 3.69%), which was significantly higher than those from the recombinant strains GG799(pKLAC1-Phs\textsubscript{mnp}) (35.55 ± 3.30%) and GG799(pKLAC1-Plo\textsubscript{mnp}) (40.02 ± 1.77%) (p < 0.05).

**Optimization of Inducible Expression for GG799(pKLAC1-Phc\textsubscript{mnp}) and the Degradation Efficiency of AFB1**

The influence of factors such as induction temperature, time, and rotation speed on degradation ratios of AFB1 was designed to analyze the expression level for the enzyme PhcMnp. As shown in Supplementary Figures 3, 4, the degradation ratios observed by the recombinants’ supernatants on AFB1 under the conditions of different induction factors had similar trends. With the increase in induction temperature, time, rotation speed, medium pH (Supplementary Figures 3A–D), and the increased concentrations of hemin, MnSO\textsubscript{4}, and galactose (Supplementary Figures 4A–C), the degradation ratios of AFB1 showed a trend of increasing at first and then decreasing.

In Supplementary Figure 4A, a high concentration of hemin inhibited the enzyme activity and degradation, causing low degradation efficiency of AFB1, which was similar to the previous results (Jiang et al., 2008).

The results in Supplementary Figure 4B indicated that with the increase in MnSO\textsubscript{4} concentration in the medium, the degradation effect on AFB1 increased at first and then decreased, and the highest degradation ratio of AFB1 reached 58.32 ± 2.07% at the MnSO\textsubscript{4} concentration of 1.0 mmol/l. Meanwhile, the expression level of the enzyme GG799(pKLAC1-Phc\textsubscript{mnp}) was also influenced by the concentration of the inducer galactose (Xia et al., 2021). In Supplementary Figure 4C, the highest degradation ratio of AFB1 reached 53.48 ± 1.63% at the MnSO\textsubscript{4} concentration of 60 g/l.

From the above results, the optimal conditions for the inducible expression of the AFB1 degradation enzyme were as follows: YEPG medium containing 60.0 g/l galactose, 1.0 mmol/l MnSO\textsubscript{4}, 1.0 mmol/l hemin, and an initial medium pH of 6.0, with induction at 30°C and 200 rpm for 96 h.

To further explore the optimal combination of the induction expression conditions of GG799(pKLAC1-Phc\textsubscript{mnp}), factors like induction temperature, induction time, rotation speed, hemin concentration, initial pH of the medium, MnSO\textsubscript{4} concentration, and galactose concentration were designed in an orthogonal experiment, and the optimization results were characterized by the degradation ratio of AFB1. The results are shown in Supplementary Table 4. The optimum induction parameter combination was A\textsubscript{2}D\textsubscript{2}B\textsubscript{2}C\textsubscript{2}E\textsubscript{1}G\textsubscript{1}. Under this condition, the highest degradation ratio of AFB1 by the recombinant’s supernatant was 67.40 ± 0.74%, which was increased by 15.6% compared to the optimal result of the single-factor optimization test.

**Optimization of Reaction Parameters for AFB1 Degradation and Determination of the Results**

In this study, the reaction conditions for AFB1 degradation by supernatant of GG799(pKLAC1-Phc\textsubscript{mnp}) were optimized with the following factors: reaction time, reaction temperature, pH, protein concentration, MnSO\textsubscript{4} concentration, glucose addition, and glucose oxidase addition, and the results are shown in Figures 3, 4. As shown in Figure 3, the trends of the degradation ratio of AFB1 under different reaction parameters were approximately similar. With the increase in induction time, temperature, pH, and protein concentration (Figures 3A–D),
the degradation ratio of AFB₁ by PhcMnp showed a trend of increasing at first and then decreasing.

As shown in Figure 4A, a high concentration of MnSO₄ in the medium inhibited the degradation ratio of AFB₁. The highest degradation ratio of AFB₁ by the recombinant's supernatant reached 61.16 ± 1.09% at the MnSO₄ concentration of 1.0 mmol/l; however, when the MnSO₄ concentration was 10.0 mmol/l, the degradation ratios of AFB₁ were less than 10%. The degradation of AFB₁ by the enzyme requires the participation of H₂O₂, but due to the instability of H₂O₂ and the reversible inactivation of manganese peroxidase under the condition of a high concentration of H₂O₂ (Wariishi et al., 1988),
we chose to add glucose and glucose oxidase to the reaction system to release H$_2$O$_2$ slowly and steadily in this study, as shown in Figures 4B,C. In Figure 4B, the degradation ratio of AFB$_1$ increased with the addition of glucose and then decreased when the glucose addition was greater than 2.5 mmol/l, probably because the H$_2$O$_2$ produced by the catalysis of 1.5 U/ml glucose oxidase at the beginning of the addition was not sufficient for the degradation of 2.0 µg/ml AFB$_1$. Conversely, when the glucose addition reached 2.5 mmol/l, the amount of H$_2$O$_2$ produced by the catalysis of 1.5 U/ml glucose oxidase was saturated.

From the above results, the optimal reaction parameters for AFB$_1$ degradation by GG799(pKLAC1-Phc$_{mnp}$) supernatants were as follows: degradation system containing 6.0 g/l protein, 1.0 mmol/l MnSO$_4$, 3.5 mmol/l glucose, and 1.2 U/ml glucose oxidase, pH of 4.5, with induction at 40°C for 36 h.

To further explore the optimal combination of the induction reaction parameters of GG799(pKLAC1-Phc$_{mnp}$), factors were designed in an orthogonal experiment, and the optimization results were characterized by the degradation ratio of AFB$_1$. The orthogonal experimental results are shown in Supplementary Table 5. The optimum induction proposal was C$_2$B$_2$D$_2$E$_2$F$_2$G$_3$. The highest degradation ratio of AFB$_1$ by the recombinant’s supernatant was 75.71 ± 1.21% under this optimum induction proposal, which was increased by 9.1% compared to the optimal result of the single-factor optimization test.

**Analysis of AFB$_1$ Degradation Products by GG799(pKLAC1-Phc$_{mnp}$) Supernatants**

The AFB$_1$ degradation products treated with fermentation broth of GG799(pKLAC1-Phc$_{mnp}$) were identified by the mass spectrum, and the results are shown in Figure 5. The chromatogram of AFB$_1$-degraded results by GG799(pKLAC1-Phc$_{mnp}$) is shown in Figure 5A, in which the AFB$_1$ retention time was found at 6.33 min. In addition, a large number of chromatogram peaks were found near the retention time of 11.15 min, which was considered to be the retention time of AFB$_1$-8,9-dihydrodiol. This compound was regarded as the major degradation product of AFB$_1$ treated with GG799(pKLAC1-Phc$_{mnp}$) supernatants. To confirm this, the AFB$_1$-8,9-dihydrodiol standards were subjected to UPLC-TQD analysis, and the retention time of this compound was found near 11.15 min (Figure 5B), whose result was consistent with that shown in Figure 5A. Theoretically, the molecular weights of AFB$_1$ and AFB$_1$-8,9-dihydrodiol are 312.27 and 346.288, respectively. The mass spectra of the samples of AFB$_1$ treated with the supernatant of recombinant GG799 (pKLAC1-Phc$_{mnp}$) in MRM scan mode are shown in Figure 5C, the [M + H]$^+$ ions with a mass-to-charge ratio (m/z) of 313 were AFB$_1$, and those slightly larger than 346 were presumed to be AFB$_1$-8,9-dihydrodiol, which was consistent with the conclusion of Wang et al. (2011), who found that AFB$_1$-8,9-dihydrodiol produced by the degradation of AFB$_1$ was much less toxic than AFB$_1$. The schematic for the degradation of AFB$_1$ by manganese peroxidases is shown in Figure 6.

**Degradation Efficiency of AFB$_1$ in Peanuts**

To investigate the degradation ratio of GG799(pKLAC1-Phc$_{mnp}$) supernatant in the actual samples, degradation experiments were conducted using peanut samples added with AFB$_1$. The degradation results are shown in Table 2. The recombinant strain GG799(pKLAC1-Phc$_{mnp}$) supernatant degraded more than 90% of AFB$_1$ in the peanut samples after twice treatments, with no significant differences (p > 0.05). When the AFB$_1$ concentration was 50.0 µg/kg, the standard deviation was larger due to the low AFB$_1$ content in the contaminated peanut samples. Besides, the larger the AFB$_1$ concentration used, the smaller the standard deviation was found. These consistent data indicated that the GG799(pKLAC1-Phc$_{mnp}$) culture supernatants were suitable for practical usage.

**Mutagenesis Site Prediction of the Manganese Peroxidase PhcMnp**

Single variants of PhcMnp were predicted based on the consensus approach, folding free energy calculations, and some structural considerations. On the one hand, amino acid evolutionary conservation distribution (Figure 7A) was obtained as follows: the amino acid sequence of PhcMnp was used to identify the homologous sequences (>40% sequence identity) from the NCBI database. After removing the redundant sequences through the CD-HIT program (Kato and Standley, 2013). The amino acid distributions at each position corresponding to that of PhcMnp were analyzed by WebLogo. On the other hand, we constructed a structural model of PhcMnp (Figure 7B) based on the crystal structures of its homolog enzyme MnP-CdII (PDB ID: 1YYG, 1.6 Å, 84% identity to PhcMnp). The software YASARA was used to perform the homology modeling. Alanine-scanning mutagenesis (Chauhan, 2017) was carried out on the homology model of PhcMnp using the Calculate Mutation Energy (Stability) protocol of Discovery Studio 2020 Client. Mutation energy prediction showed that six variants (70H, 110S, 174K, 178R, 192S, and 243T) had a much lower energy than others. These sites were then selected for full mutation scanning. Considering the conservation and energy, it was reasoned that the seven single variants (H70D, S110A, K174S, R178E, S192A, T243Q, and T243P) may have potentially enhanced activity. Besides, based on the criteria of non-mutation of highly conservative amino acids, low mutation energy, and being close to heme (<5 Å), it was reasoned that the 13 single variants (E59A, I65L, N105G, N105A, N105L, L138V, F179M, V199I, R201A, K204V, K204E, V205I, and L300M) may also have potentially enhanced activity.

**DISCUSSION**

Manganese peroxidase is of increasing interest due to its potential for industrial applications, which was originally used in the...
FIGURE 5 | Analysis of the degradation products of AFB$_1$ by GG799(pKLAC1-Phcmnp) fermentation broth. (A) Chromatogram of AFB$_1$ metabolites degraded by fermentation broth of GG799(pKLAC1-Phcmnp). The chromatogram peak at retention time of 6.3 min was AFB$_1$. (B) Chromatogram of the AFB$_1$-8,9-dihydrodiol standard. The chromatogram peak near the retention time of 11.19 min was AFB$_1$-8,9-dihydrodiol standard. (C) Mass spectrum of AFB$_1$ degradation product by fermentation supernatants of GG799(pKLAC1-Phcmnp). [M + H]$^+$ ions with a mass-to-charge ratio (m/z) of 313 (circled) were AFB$_1$, and those of slightly larger than 346 (circled) were presumed to be AFB$_1$-8,9-dihydrodiol.
and then complex I is reduced by Mn$^{2+}$ manganese peroxidase. Some reports (Xia et al., 2021) showed that the presence of excessive Mn$^{2+}$ may also inhibit the enzymatic activity of manganese peroxidase. As for the degradation of AFB$_1$, the expression level for the enzyme PhcMnp is mainly influenced by factors such as induction temperature, time, and rotation speed; meanwhile in this work, the inducible expression for the enzyme PhcMnp was also regulated by the concentration of hemin and Mn$^{2+}$. Hemin is a non-covalently bound prosthetic group in MnP, which is crucial to activating soluble MnPs (Wang et al., 2019). Also, manganese peroxidase consists of a ferric heme group and Mn$^{2+}$ as its active center, and previous reports (Whitwam and Tien, 1996; Gettemy et al., 1998) showed that the manganese peroxidase genes are regulated by Mn$^{2+}$ at the transcriptional level, and a certain amount of Mn$^{2+}$ may promote the synthesis of manganese peroxidase.

As for the degradation of AFB$_1$, the catalytic reaction of manganese peroxidase (MnP) requires the participation of H$_2$O$_2$ and Mn$^{2+}$. The Fe$^{3+}$-containing MnP is oxidized by H$_2$O$_2$ to produce complex I (Fe$^{4+}$-oxygen–porphyrin radical complex), and then complex I is reduced by Mn$^{2+}$ to produce complex II (Fe$^{4+}$-oxygen–porphyrin complex) by a single electron. Finally, complex II is reconverted to MnP and Mn$^{3+}$ by using Mn$^{2+}$ as an electron donor (Hofrichter, 2002). The product Mn$^{3+}$ can form chelates with some organic acids (e.g., oxalic acid, malonic acid, and malic acid) to oxidize complex organics to achieve the degradation of AFB$_1$ (Wang et al., 2019). Therefore, the addition of Mn$^{2+}$ in the reaction system is necessary, but some reports (Xia et al., 2021) showed that the presence of excessive Mn$^{2+}$ may also inhibit the enzymatic activity of manganese peroxidase.

The degradation product of AFB$_1$ was analyzed and determined to be AFB$_1$-8,9-dihydrodiol in this work. The main pathway of degradation of AFB$_1$ was through the free radicals generated by the interaction of oxidized Mn$^{3+}$ with the dicarboxylic acid malonate (Wang et al., 2019), which increased the redox potential for oxidation of AFB$_1$. In the degradation process, the intermediate AFB$_1$-8,9-epoxide was produced before AFB$_1$-8, 9-dihydrodiol, and it would be rapidly hydrolyzed to AFB$_1$-8,9-dihydrodiol. The degradation product AFB$_1$-8,9-dihydrodiol was greatly less toxic than AFB$_1$, since it has no binding sites for DNA, lipids, and other macromolecules, thus achieving the purpose of detoxification of AFB$_1$ (Wang et al., 2019).

As for the degradation ratio of AFB$_1$, the recombinant laccase expressed in Aspergillus niger (118.0 U/l) achieved 55% of the degradation ratio for AFB$_1$ (Alberts et al., 2009), and manganese peroxidase produced by Phanerochaete sordida YK-624 achieved 86% of the degradation ratio for AFB$_1$ (Wang et al., 2011). Doyle and Marth (1979) found that lactoperoxidase in Aspergillus parasiticus could degrade AFB$_1$ with up to 30.4% degradation ratio. However, the degradation ratio of AFB$_1$ in the peanuts by the manganese peroxidase in this work reached more than 90%, which was much higher than those results by the enzymes produced in wild-type strains.

In conclusion, the manganese peroxidases used in this study were all derived from food-safe microorganisms (Phanerochaete sordida, Pleurotus ostreatus, and Phanerochaete chrysosporium) and the recombinant enzyme PhcMnp performed well in the degradation of AFB$_1$. The food-grade expression system including the host K. lactis GG799 and the vector pKLAC1 was used in this work, in which the yeast K. lactis has been widely used in the food, feed, and pharmaceutical industries without

### Table 2: Degradation results of AFB$_1$ in peanut samples by GG799(pKLAC1-PhcMnp) culture supernatants.

| Experiment number | Concentration of AFB$_1$ added in samples (µ g/kg) | Concentration of AFB$_1$ detected in samples (µ g/kg) | Concentration of AFB$_1$ detected after once treatment (µ g/kg) | Degradation ratio after once treatment (%) | Concentration of AFB$_1$ detected after twice treatments (µ g/kg) | Degradation ratio after twice treatments (%) |
|-------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| 1                 | 50.0                       | 43.77                           | 7.59                             | 82.66 ± 13.17                  | –                               | *82.66 ± 13.17                  |
| 2                 | 500.0                      | 417.96                          | 98.31                            | 76.48 ± 1.99                   | 27.67                           | 93.38 ± 0.93                   |
| 3                 | 2000.0                     | 1766.39                         | 368.26                           | 79.15 ± 0.07                   | 146.39                          | 91.71 ± 3.99                   |

Three parallel reactions were done in each group, and the standard deviations were listed. *Degradation ratio after the second treatment in experiment number 1 followed the first degradation ratio.
environmental risks (Spohner et al., 2016). Therefore, this study provides a basis for subsequent large-scale fermentation of food-grade recombinant enzymes for degradation of mycotoxins in foodstuffs or feedstuffs. Furthermore, results showed that the enzyme PhcMnp has potential for practical application in the food industry. Besides, mutagenesis site prediction of the manganese peroxidase PhcMnp provided the theoretical basis for the following experiments in improvement or property modification of the enzyme.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

YX conceived the research strategies, designed the experiments, gave important guidance to the experiments, and assisted in completing the final manuscript. RH designed the experiments, conducted the experiments, and prepared the drafts of the manuscript. YS conducted the experiments and analyzed the data. HZ conducted the protein structure analysis. MG participated in the mycotoxin degradation experiments in practical examples. XH, XC, and QC provided partial materials, investigation
data, and some experimental technique supports. ZW provided guidance and partial financial supports for the experiments. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.821230/full#supplementary-material

REFERENCES

Abrar, M., Anjum, F. M., Butt, M. S., Pasha, I., Randhawa, M. A., Saeed, F., et al. (2013). Aflatoxins: biosynthesis, occurrence, toxicity, and remedies. Crit. Rev. Food Sci. Tech. 53, 862–874. doi: 10.1080/01448194.2011.563154

Alberts, J. F., Gelderblom, W., Botha, A., and Zyl, W. (2009). Degradation of aflatoxin B1 by fungal laccase enzymes. Int. J. Food Microbiol. 135, 47–52. doi: 10.1016/j.ijfoodmicro.2009.07.022

Assaf, J. C., Nahle, S., Chokr, A., Louka, N., Atoui, A., and El Khoury, A. (2019). Assorted methods for decontamination of Aflatoxin M1 in milk using microbial adsorbents. Toxins (Basel) 11:304. doi: 10.3390/toxins11060304

Cao, H., Liu, D., Mo, X., Xie, C., and Yao, D. (2011). A fungal enzyme that degrade aflatoxin. China (31871881).

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Gowda, N. K. S., Suganthi, R. U., Malathi, V., and Raghavendra, A. (2007). Efficacy of heat treatment and sun drying of aflatoxin-contaminated feed for reducing the harmful biological effects in sheep. Anim. Feed Sci. Tech. 133, 167–175. doi: 10.1016/j.anipts.2006.08.009

Ha, H. C., Honda, Y., Watanabe, T., and Kuwahara, M. (2001). Production of manganese peroxidase by pellet culture of the lignin-degrading basidiomycete, Pleurotus ostreatus. Appl. Microbiol. Biotechnol. 55, 704–711. doi: 10.1007/s002530100653

Haque, M. A., Wang, Y., Shen, Z., Li, X., Saleemi, M. K., and He, C. (2020). Mycotoxin contamination and control strategy in human, domestic animal and poultry: a review. Microb. Pathog. 142:104095. doi: 10.1016/j.micpath.2020.104095

Hofrichter, M. (2002). Review lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. 30, 454–466. doi: 10.1016/S0141-0229(01)00528-2

Huang, Y., Niu, B. F., Gao, Y., Fu, L. M., and Li, W. Z. (2010). CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26, 680–682. doi: 10.1093/bioinformatics/btp003

Ismail, A., Goncalves, B. L., de Neef, D. V., Zonilacaqu, B., Coppa, C., Hintzsche, H., et al. (2018). Aflatoxin in foodstuffs: occurrence and recent advances in decontamination. Food Res. Int. 113, 74–85. doi: 10.1016/j.foodres.2018.06.067

Jiang, F., Kongaeree, P., Charron, R., Lajoie, C., Xu, H., Scott, G., et al. (2008). Production and separation of manganese peroxidase from heme amended yeast cultures. Biotechnol. Bioeng. 99, 540–549. doi: 10.1002/bit.21590

Katok, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. doi: 10.1093/molbev/mso101

Loi, M., Fanelli, F., Liuzzi, V. C., Logrieco, A. F., and Mule, G. (2017). Mycotoxin biotransformation by native and commercial enzymes: present and future perspectives. Toxins (Basel) 9:111. doi: 10.3390/toxins9040111

Loi, M., Fanelli, F., Zucca, P., Liuzzi, V. C., Quintieri, L., Cimmarusti, M. T., et al. (2016). Aflatoxin B1 and M1 degradation by Lac2 from Pleurotus pulmonarius and relocator meditoxins. Toxins (Basel) 8:245. doi: 10.3390/toxins8090245

Marques, M. M., de Gonzalez, A. B., Beland, F. A., Browne, P., Demers, P. A., Lachennier, D. W., et al. (2019). Advisory group recommendations on priorities for the IARC monographs. Lancet Oncol. 20, 763–764. doi: 10.1016/s1470-2045(19)30246-3

Peng, Z., Chen, L., Zhu, Y., Huang, Y., Hu, X., Wu, Q., et al. (2018). Current major degradation methods for aflatoxins: a review. Trends Food Sci. Technol. 80, 155–166. doi: 10.1016/j.tifs.2018.08.009

Pitt, J. I., and Miller, J. D. (2017). A concise history of mycotoxin research. J. Agr. Food Chem. 65, 7021–7033. doi: 10.1021/acs.jafc.6b04494

Spohn, S. C., Schaum, V., Quitmann, H., and Czermak, P. (2016). Kluyveromyces lactis: an emerging tool in biotechnology. J. Biotechnol. 222, 104–116. doi: 10.1016/j.jbiotec.2016.02.023

Styriak, I., Konkovd, E., Kmec, V., Bbhm, J., and Razzazi, E. (2001). The use of yeast for microbial degradation of some selected mycotoxins. Mycotoxin Rev. 17, 24–27. doi: 10.1016/S1541-4337(19)30246-3

Tarazi, S., Ahmadi, S., Ostvar, N., Ghafouri, H., Sarikhan, S., Mahmoodi, Z., et al. (2021). Enhanced soluble expression of glutathione S-transferase Mu from
Rutilus kutum by co-expression with Hsp70 and introducing a novel inhibitor for its activity. Process Biochem. 111, 261–266. doi: 10.1016/j.procbio.2021.10.003

van den Dungen, M. W., Boer, R., Wilms, L. C., Efimova, Y., and Abbas, H. E. (2021). The safety of a Kluyveromyces lactis strain lineage for enzyme production. Regul. Toxicol. Pharm. 126:105027. doi: 10.1016/j.yrtph.2021.10.002

von Hertwig, A. M., Iamanaka, B. T., Amorim Neto, D. P., Rezende, J. B., Martins, L. M., Taniwaki, M. H., et al. (2020). Interaction of Aspergillus flavus and A. parasiticus with Salmonella spp. isolated from peanuts. Int. J. Food Microbiol. 328:108666. doi: 10.1016/j.ijfoodmicro.2020.108666

Wang, J. Q., Ogata, M., Hirai, H., and Kawagishi, H. (2011). Detoxification of aflatoxin B1 by manganese peroxidase from the white-rot fungus Phanerochaete sordida YK-624. FEBS Microbiol. Lett. 314, 164–169. doi: 10.1111/j.1574-6968.2010.02158.x

Wang, L., Luo, Y., Luo, X., Wang, R., Li, Y., Li, Y., et al. (2016a). Effect of deoxynivalenol detoxification by ozone treatment in wheat grains. Food Control 66, 137–144. doi: 10.1016/j.foodcont.2016.01.038

Wang, L., Shao, H., Luo, X., Wang, R., Li, Y., Li, Y., et al. (2016b). Effect of ozone treatment on deoxynivalenol and wheat quality. PLoS One 11:e0147613. doi: 10.1371/journal.pone.0147613

Wang, X., Qin, X., Hao, Z., Luo, H., Yao, B., and Su, X. (2019). Degradation of four major mycotoxins by eight manganese peroxidases in presence of a dicarboxylic acid. Toxins (Basel) 11:566. doi: 10.3390/toxins11100566

Wang, Y., Zhao, C., Zhang, D., Zhao, M., Zheng, D., Peng, M., et al. (2018). Simultaneous degradation of aflatoxin B1 and zearalenone by a microbial consortium. Toxicicon 146, 69–76. doi: 10.1016/j.toxicicon.2018.04.007

Wariishi, H., Akileswaran, L., and Gold, M. H. (1988). Manganese peroxidase from the basidiomycete Phanerochaete chrysosporium: spectral characterization of the oxidized states and the catalytic cycle. Biochemistry 27, 5365–5370. doi: 10.1021/bi00414a061

Whitwam, R., and Tien, M. (1996). Heterologous expression and reconstitution of fungal Mn peroxidase. Arch. Biochem. Biophys. 333, 439–446. doi: 10.1006/abbi.1996.0413

Xia, Y., Wu, Z., He, R., Gao, Y., Qiu, Y., Cheng, Q., et al. (2021). Simultaneous degradation of two mycotoxins enabled by a fusion enzyme in food-grade recombinant Kluyveromyces lactis. Bioresour. Bioprocess. 8:62. doi: 10.1186/s40643-021-00395-1

Yang, B., Zhang, C., Zhang, X., Wang, G., Li, L., Geng, H., et al. (2020). Survey of aflatoxin B1 and heavy metal contamination in peanut and peanut soil in China during 2017–2018. Food Control 118:107372. doi: 10.1016/j.foodcont.2020.107372

Yehia, R. S. (2014). Aflatoxin detoxification by manganese peroxidase purified from Pleurotus ostreatus. Braz. J. Microbiol. 45, 127–133. doi: 10.1590/s1517-83822014005000026

Zhang, W., Liu, Y., Liang, B., Zhang, Y., Zhong, X., Luo, X., et al. (2020). Probabilistic risk assessment of dietary exposure to aflatoxin B1 in Guangzhou, China. Sci. Rep. 10:7973. doi: 10.1038/s41598-020-64295-8

Zhao, M., Wang, X. Y., Xu, S. H., Yuan, G. Q., Shi, X. J., and Liang, Z. H. (2020). Degradation of ochratoxin A by supernatant and ochratoxinase of Aspergillus niger W-35 isolated from cereals. World Mycotoxin J. 13, 287–298. doi: 10.3920/wmj2019.2446

Conflict of Interest: XC is employed by Anhui Heiwa Food Technology Co., Ltd.

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