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Research Article 研究报告

Characterization of the aflatoxin B1 degradation role of laccases in *Stenotrophomonas acidaminiphila*

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Abstract: [Objective] Characterization of the aflatoxin B1 (AFB1) degradation roles of the laccases screened from *Stenotrophomonas acidaminiphila* CW117. **[Methods]** Two laccase genes *lc1* and *lc2* from strain CW117 genome were screened, and their AFB1 degrading activity was examined *in vitro* by heterologous expressed proteins of rLC1 and rLC2 in *E. coli* BL21. On the basis of *in vitro* test, two laccase-deficient strains CW117$^{\Delta lc1}$and CW117$^{\Delta lc1\text{-}lc2}$ were constructed by homologous recombination method by using suicide plasmid pK18*mobsacB*, and the laccases (*lc1* and *lc2*) AFB1 degradation role on strain CW117 was validation *in vivo*. **[Results]** Laccase rLC1 showed the AFB1 degradation activity *in vitro*, but rLC2 did not show degradation activity. Degradation activity of rLC1 was improved by redox mediators of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid), acetosyringone or syringaldehyde. The degrading activity of mutants CW117$^{\Delta lc1}$ and CW117$^{\Delta lc1\text{-}lc2}$ showed similar degradation activity to the wild-type strain CW117 in most evaluation time-points. **[Conclusion]** Laccase LC1 from *S. acidaminiphila* showed AFB1 degradation activity, and the degradation activity could be enhanced by redox mediators as previous study. However, the laccases' contribution to AFB1 degradation in strain CW117 was minimal, and other degradation pathways existed in the strain.

Keywords: detoxification, laccase, aflatoxin B1, degradation gene, mutant

The aflatoxins' chemical structures consist of a coumarin and bifuran rings; the natural contaminants are mainly produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*[1]. As well-known, aflatoxin B1 (AFB1) is recognized as the most potent and prevalent aflatoxin; the extremely toxic effects of mutagenic, carcinogenic and teratogenic on humans and livestock pose a serious health and economic

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hazards[2–3].

At present, various microbial species (or genera) have been isolated and characterized for aflatoxins degradation, and the microbial transformation (or degradation) has been considered as one of the most effective means to aflatoxins detoxification. The most investigated microbial species including *Armillaria* spp.[4–6], *Pleurotus* spp.[7], *Pseudomonas* spp.[8], *Bacillus* spp.[9–11], *Flavobacterium* spp.[12–13], *Mycobacterium* spp.[14–15], *Rhodococcus* spp.[15–17] and *Stenotrophomonas* spp.[18]. Besides the degrading microbial strains characterization, aflatoxins degrading enzymes (including degrading genes) equally received great attention. For instance, manganese peroxidase (MnP)[19], aflatoxin-oxidase (AFO)[6] and pyridoxamine 5′-phosphate oxidases (PNPOxs)[20] were determined as aflatoxins detoxification enzymes. Among these aflatoxins detoxification enzymes from microbial strains, the laccases were the most focused and recognized degrading agent for aflatoxin detoxification[21–23]. Laccases belong to the superfamily of multicopper oxidases and widely present in bacteria and fungi[24–26], and laccase catalyze ring cleavage of aromatic compounds, and are generally considered as an important biotransformation candidate to environmental pollutants[27]. In previous studies, laccases from *Pleurotus pulmonarius*[21], *Pleurotus eryngii*[22–23] and *Trametes versicolor*[28] were determined as the aflatoxin detoxification agents. After degraded by laccases from the microbial species, the biotoxicities (or mutagenicity) of degraded products were significantly reduced or even disappeared[29]. Degradation results in previous studies indicated that laccases were the general recognized detoxification agent to aflatoxin degradation.

Our previous study found that strain *S. acidaminiphila* CW117 showed efficient degrading activity to aflatoxin B1, and the 24 h degrading ratio higher than 85%. One laccase gene *lc1* from strain CW117 were screened and heterologous expressed by *E. coli* BL21. However, AFB1 degrading test showed that rLC1 showed relatively low degrading activity *in vitro*[30]. In view of the results, the following possibilities might be included: (i) laccases were the sole bioagents (enzymes) for AFB1 transfomation, but the heterologously produced rLC1 proteins in *E. coli* system were not appropriate folded (or mismodified) and resulted in low degradation activity; (ii) Other than laccases, some cofactors (*e.g.*, coenzymes or redox mediators) in strain CW117 can significantly assist the degradation ability of laccases, but the cofactors (or coenzymes) are absent in *E. coli* system; (iii) Other efficient degrading enzymes (or other degrading agents) are responsible for AFB1 degradation, and laccases were only a secondary degradation agent in strain CW117. In this study, other laccase genes were further screened, and the AFB1 degradation characteristics of all laccase genes were investigated by methods of *in vitro* and *in vivo*, and the laccase degradation role was examined.

1 Materials and methods

1.1 Chemicals, bacterial strains and plasmids

The AFB1 degrading strain *S. acidaminiphila* CW117 was previous isolated and preserved in our laboratory. AFB1 standard was obtained from Pribolab Pte. Ltd (Bejing, China), other analytical reagents were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Chromatographically grade solvents (*i.e.*, methanol, acetonitrile) were purchased from TEDIA (Shanghai, China). The analytical grade (purity>98%) of ABTS [2,2-azino-bis-(3-ethyl benzothiazoline-6-sulphonic acid)], AS (acetosyringone) and SA (syringaldehyde) were obtained from Sigma-Aldrich (Shanghai, China). The strains, plasmids and the PCR primers used in this study are shown in Table 1. The antibiotics (*i.e.*, gentamycin and kanamycin) used for mutant screening were purchased from Sangon Biotech Co.,

Ltd. (Shanghai, China), and the antibiotic solutions were prepared by diluting in distilled water at the final concentration of 50 μg/mL for each. Bacterial culture media of Luria-Bertani (LB), nutrient broth (NB) and nutrient agar (NA) are the products of Difco (Lawrence, Kansas, USA). The affinity chromatography resin (*ProteIniso*® GST Resin) used for heterologous expressed protein purification is the TransGen Biotech (Beijing, China) product. The DNA polymerase (PrimerSTAR Max DNA Polymerase), restriction endonuclease (*Xba* Ⅰ and *Hin*d Ⅲ) and DNA ligase (solution Ⅰ) used in this study are the products of TaKaRa (Dalian, China). Agarose gel DNA recovery kit is the Tiangen (Beijing China) product, and the plasmid MiniPrep kit is product of AxyPrep (Axygen, CA, USA).

1.2 Laccase genes cloning, expression and AFB1 degradation

During genome sequence analysis, two laccase genes (*i.e.*, *lc1* and *lc2*) were screened from complete genome sequence (NZ_CP012900.1) of strain *S. acidaminiphila* ZAC14D2_NAIMI4_2 which showed the closest taxonomic affiliation to strain CW117. Laccase genes were amplified by using CW117 genomic DNA and the primer pairs of *lc1*-F/R and *lc2*-F/R with PrimerSTAR Max DNA Polymerase (Table 1). The PCR condition was 5 min denaturing at 98 °C, followed by 30 cycles of 5 s denaturing at 98 °C, 5 s annealing at 61 °C, 5 s extension at 72 °C, and a final 10 min extension at 72 °C. The purified

Table 1. Thestrains, plasmids and primers used in this study

Strains, plasmids or primers	Characterization	Reference or restriction sites
Strain CW117		
pGEX/*lc1*	LC1 expression strain	This study
pGEX/*lc2*	LC2 expression strain	This study
CW117$^{\Delta lc1}$	The gene *lc1* deletion mutant of CW117	This study
CW117$^{\Delta lc1\text{-}lc2}$	The gene *lc1* and *lc2* deletion mutant of CW117	This study
Escherichia coli Trans1-T1 phage resistant	Receptive cells	TransGen Biotech, Beijing, China
Escherichia coli BL21	Expression cells	TransGen Biotech, Beijing, China
pK18*mobsacB*	Allelic exchange vector, Kan^R	[40]
lc1-F*	CGGGATCCATGGCCGCCGCGTTGCC	*Bam*H Ⅰ
lc1-R	CCGCTCGAGTCACCGCGCCATCCACAC	*Xho* Ⅰ
lc2-F	CGGAATTCATGAAAAACGATTTCCTTTCCG	*Eco*R Ⅰ
lc2-R	CCGCTCGAGTCATGCCTCGATCCTCACT	*Xho* Ⅰ
lc1-US-F	GCTCTAGAGCGTCGGTATCGGTATGCG	*Xba* Ⅰ
lc1-US-R	ACTGGCGGGTGGACCTGTA	
lc1-DS-F#	gtacaggtccacccgccagtGCCCAGGTTGAAACTGTCG	Lowercase indicates adapter sequence for overlapping PCR
lc1-DS-R	CCCAAGCTTATCCCGCTGTCGGTCCTGT	*Hin*d Ⅲ
ver1-F	CCTGCCCGGCCTAATCCA	*lc1* deletion identification primer
ver1-R	TCCCGCCTGAGCGAATGG	*lc1* deletion identification primer
lc2-US-F	GCTCTAGACCGGAATCAGGTGGGTATCG	*Xba* Ⅰ
lc2-US-R	GGGAACTCCATCGGCCGC	
lc2-DS-F	gggcggccgatggagttcccAGACGACGACCCGATCCCT	lowercase indicates adapter sequence for overlapping PCR
lc2-DS-R	CCCAAGCTTCGCGTCCAGCGACCACTC	*Hin*d Ⅲ
ver2-F	CGGGTGGCTGGACTCGT	*lc2* deletion identification primer
ver2-R	CACCACGGTGAGATGGAATG	*lc2* deletion identification primer

*Underlined sequences represent the digestion site.

PCR product and expression plasmid pGEX-4T-1 were digested by *Bam*HⅠ (or *Eco*RⅠ) and *Xho*Ⅰ at 37 °C overnight, respectively. The digest mixture contained 1.0 μL *Xho*Ⅰ, 1.0 μL *Bam*HⅠ (or *Eco*RⅠ), 1 μg of purified *lc1* or *lc2* (or 1 μg pGEX-4T-1) and 5 μL of 10×H buffer (TaKaRa, Dalian, China), and then supplemented distilled water to 50 μL. The digested *lc1* (or *lc2*) fragment and expression plasmid was purified using an agarose gel DNA recovery kit according to manufacturer's instructions, and the purified *lc1* or *lc2* fragment was ligated to digested pGEX-4T-1 by incubation with solution Ⅰ DNA ligase for 1 h at 16 °C. The ligation mixture consists of 2 μL digested pGEX-4T-1, 4 μL digested *lc1* or *lc2* and 6 μL solution Ⅰ DNA ligase. After ligation, 5 μL of the recombinant plasmid (pGEX/*lc1* or pGEX/*lc2*) was transformed to *E. coli* Trans1-T1 by heated shock and identified by PCR sequencing. The positive clone was enriched in AmpR LB broth which containing 50 μg/mL ampicillin, and the recombinant plasmid was extracted by an Plasmid MiniPrep kit according to manufacturer's instructions and transformed to *E. coli* BL21 by heated shock.

The laccase transformant (containing pGEX/*lc1* or pGEX/*lc2*) was incubated in Amp^R LB brothat 16 °C with agitation of 150 r/min; until the culture OD_{600} reached 0.6, about 0.2 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and followed by additional 4 hours incubation. The recombinant proteins of rLC1 and rLC2 were purified by *ProteIniso* GST resin according to manufacturer's instructions. The purity of obtained rLC1 (or rLC2) recombinant protein was examined by SDS-PAGE (BioRad, Hercules, USA), and the protein concentration was evaluated by spectrophotometrically method[31]. The method of Cai *et al*.[30] was used to determine the rLCs' AFB1 degradation activity, and the method of Loi *et al*.[21,23] with minor modification was used to evaluate the redox mediators' (*i.e.*, ABTS, AS and SA) assistant effects on AFB1 degradation. Briefly, 0.1 mL of purified rLC1 (1.0 mg/mL) and 0.1 mL redox mediator (50 mmol/L) were added to 0.3 mL AFB1 working solution (40 μg/L) to make the final degradation mixture. The redox mediator and AFB1 working solution were prepared by glycine-hydrochloric acid buffer (pH 4.0). The redox mediator (without rLC1) degradation test and rLC1 (without redox mediator) degradation test were selected as controls. The degradation tubes were incubated for 24 h at 37 °C, and AFB1 residues were analyzed by using HPLC[32].

1.3 Construction of mutant $CW117^{\Delta lc1}$ and double mutant $CW117^{\Delta lc1\text{-}lc2}$

The protocols of recombinant plasmid pK18*mobsacB*_US-DS construction and the laccase mutants $CW117^{\Delta lc1}$and $CW117^{\Delta lc1\text{-}lc2}$ screening (as shown in Figure 1) were followed as the methods of previous[33–35]. The upstream (*lc1*-US) and downstream (*lc1*-DS) DNA fragments which flanking the *lc1* open reading frame (ORF) were amplified by PCR using the PrimerSTAR Max DNA Polymerase. A primer pair of *lc1*-US-F and *lc1*-US-R, and a primer pair of *lc1*-DS-F and *lc1*-DS-R were employed for *lc1*-US and *lc1*-DS in PCR amplification, respectively (Table 1). The PCR condition of *lc1*-US (or *lc1*-DS) were 5 min denaturing at 98 °C, followed by 30 cycles of 5 s denaturing at 98 °C, 5 s annealing at 61 °C, 5 s extension at 72 °C, and a final 10 min extension at 72 °C. After PCR amplification, both PCR products were purified using an agarose gel DNA recovery kit according to manufacturer's instruction. The overlap PCR was performed using the primer pair of *lc1*-US-F and *lc1*-DS-R (Table 1) with the PrimerSTAR Max DNA Polymerase (TaKaRa, Dalian, China), the purified PCR products of *lc1*-US and *lc1*-DS (1 μL for each) were selected as DNA templates. The overlap PCR conditions of *lc1*-US-DS were 5 min denaturing at 98 °C, followed

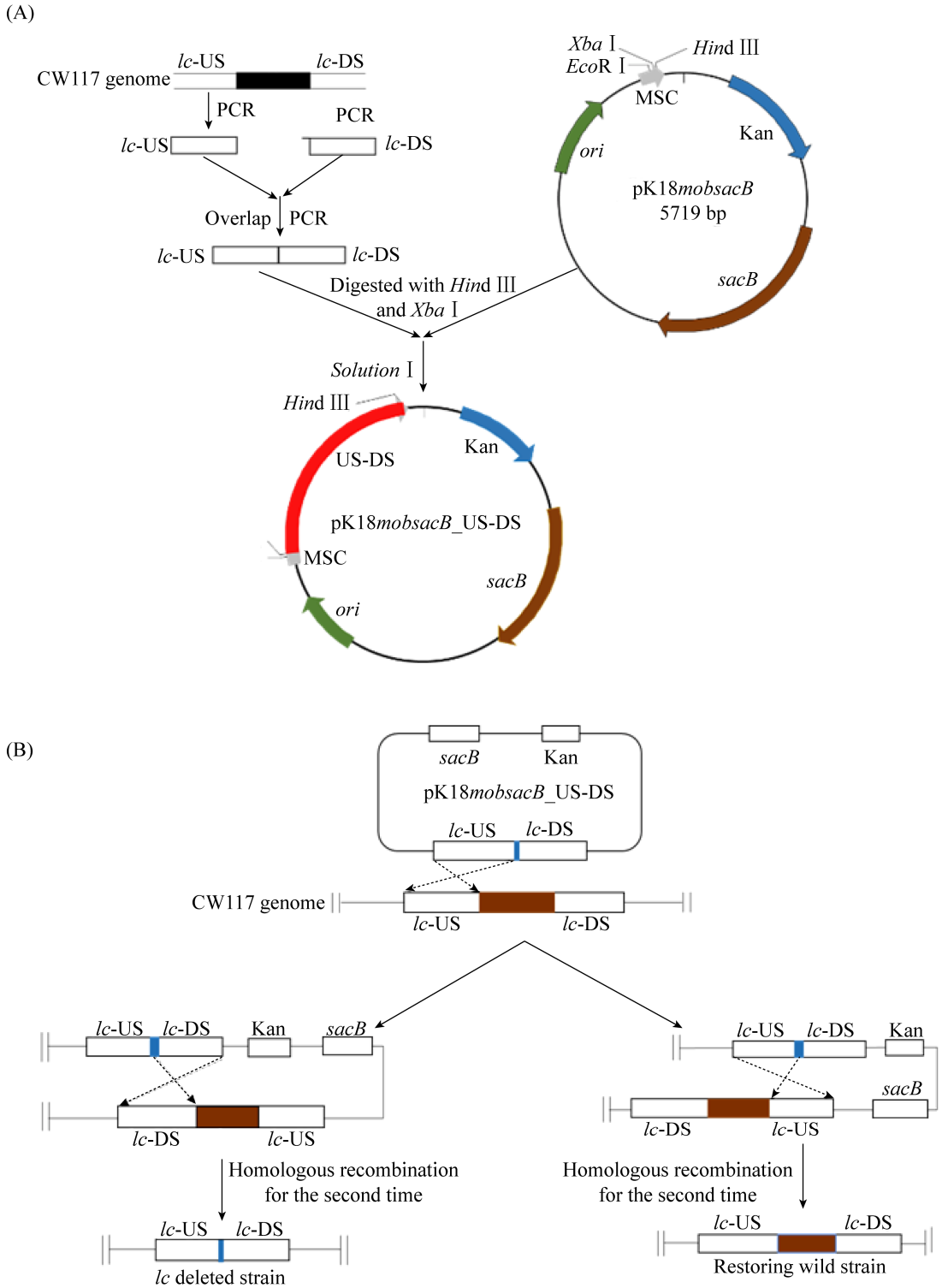

Figure 1. Schematic description of recombinant plasmid pK18*mobsacB*_US-DS construction and gene mutant screening. A: pK18*mobsacB*_US-DS construction; B: genemutant screening.

by 30 cycles of 5 s denaturing at 98 °C, 10 s annealing at 61 °C, 10 s extension at 72 °C, and a final 10 min extension at 72 °C.

The purified overlap PCR product (*lc1*-US-DS) and suicide plasmid pK18*mobsacB* were digested with *Hin*d Ⅲ and *Xba* Ⅰ at 37 °C overnight, respectively. The digest mixture contained 1.0 μL *Xba* Ⅰ, 1.0 μL *Hin*d Ⅲ, 1.0 μL purified *lc1*-US-DS (or 1 μg pK18*mobsacB*) and 5 μL of 10×M buffer (TaKaRa, Dalian, China), then supplemented distilled water to 50 μL. The digested *lc1*-US-DS fragment and suicide plasmid was purified using an agarose gel DNA recovery kit, respectively. The purified *lc1*-US-DS fragment was ligated to digested pK18*mobsacB* by incubation with solution Ⅰ DNA ligase 1 h at 16 °C. The ligation mixture consists of 2 μL digested pK18*mobsacB*, 4 μL digested *lc1*-US-DS and 6 μL solution Ⅰ DNA ligase. After ligation, 5 μL of the ligation product were transformed to *E. coli* Trans1-T1, and screened by KanR LB agar which containing 50 μg/mL kanamycin. Positive clones were identified by PCR sequencing, and the successful recombinant plasmid was defined as pK18*mobsacB_lc1*-US-DS. The positive clone was enriched by Kan^R LB medium, and extracted by a Plasmid MiniPrep kit. The recombinant plasmid pK18*mobsacB_lc1*-US-DS was transformed into strain CW117 (wild-type) by electroporation method as previous[36]. After transformation, cell solution was evenly coated on the Kan^R nutrient agar (NA). Single colony on Kan^R NA screening plates was inoculated into 5 mL Kan^R NB, and incubated at 37 °C, with agitation of 180 r/min. Until the culture OD_{600} reached 0.6, bacteria cells (0.5 mL) were collected by centrifugation (5000×*g*), and washed twice by sterile NB, and resuspended in 0.5 mL NB. The serial NB diluted bacteria suspensions were coated on the NA containing 13% sucrose (named "sucrose screening agar"), and the positive clones in sucrose plate were rescreened by Kan^R NA which containing 50 μg/mL kanamycin. The resulting colonies which grown on sucrose plate but sensitive to 50 μg/mL kanamycin were considered as the mutant candidate of $CW117^{\Delta lc1}$. The mutant candidate was identified by PCR sequencing with PrimerSTAR Max DNA Polymerase, and the primer pair of ver1-F and ver1-R (Table 1). The PCR conditions were 5 min denaturing at 98 °C, followed by 30 cycles of 5 s denaturing at 98 °C, 5 s annealing at 55 °C, 10 s extension at 72 °C, and a final 10 min extension at 72 °C.

The construction and screening protocols of double gene *lc1* and *lc2* mutant $CW117^{\Delta lc1\text{-}lc2}$ were followed the construction and screening methods of $CW117^{\Delta lc1}$, and the mutant $CW117^{\Delta lc1}$ was selected as starting strain (original strain) for $CW117^{\Delta lc1\text{-}lc2}$ construction. The PCR primer pairs of *lc2*-US (*lc2*-US-F, *lc2*-US-R), *lc2*-DS (*lc2*-DS-F, *lc2*-DS-R) and gene *lc2* deletion verification (ver2-F and ver2-R) were shown as Table 1.

1.4 The AFB1 degradation tests by wide-type strain and mutants

Strains of CW117, $CW117^{\Delta lc1}$ and $CW117^{\Delta lc1\text{-}lc2}$ were inoculated on NA, and single colonies were selected, inoculated and incubated in 5 mL NB at 37 °C, with agitation of 180 r/min. Until the culture OD_{600} absorbance reached 0.6, the fresh culture (0.5 mL) transferred to 50 mL NB containing 20 μg/L AFB1. The degradation tests were performed at 37 °C, with agitation of 180 r/min. The 50 mL NB containing 20 μg/L AFB1 without bacteria inoculation was used as control. During degradation, AFB1 residues at 0th, 12th, 24th, 36th, 48th, 60th and 72th hour were determined by HPLC following the method as previous[31].

1.5 The AFB1 degradation tests by culture supernatant of wide-type strain and mutants

Fresh culture (0.5 mL) of CW117, $CW117^{\Delta lc1}$ or $CW117^{\Delta lc1\text{-}lc2}$ was inoculated into 50 mL NB, incubated at 37 °C with agitation of 180 r/min for 48 h. The bacterial culture (CW117, $CW117^{\Delta lc1}$ or $CW117^{\Delta lc1\text{-}lc2}$) was centrifuged at 5000×*g* for 10 min

at 4 °C, and the supernatant and cell pellet were separated. Culture supernatant was prepared by passing the supernatant through a sterile filter (0.22 μm, Millipore, Billerica, USA). During the degradation test, 25 mL culture supernatant was mixed with 25 mL PBS (pH 6.8) which containing 40 μg/L AFB1.The degradation tubes were incubated at 37 °C without agitation. During degradation, AFB1 residues at 0th, 1th, 3th, 6th, 9th, 12th and 24th hour were determined by HPLC following the method as previous[31].

1.6 Data analysis

There were three biological repeats in the experimental data. One way ANOVA and *t*-test ($^{*}P<0.05$) were used to analyze the data by Graphpad prism (5.0) software.

2 Results

2.1 Laccase genes cloning, expression and protein purification

As shown in Figure 2, gene *lc1* (780 bp) was cloned by the primer pair of *lc1*-F/R and the PCR condition, and recombinant plasmid pGEX/*lc1* was successfully constructed and transformed to *E. coli* BL21. Similarly, gene *lc2* (1773 bp) was cloned by the primer pair of *lc2*-F/R and the PCR condition, and recombinant plasmid pGEX/*lc2* was also successfully constructed and transformed to *E. coli* BL21 (Figure 3). After heterologous expressed in *E. coli* BL21, the recombinant proteins of rLC1 and rLC2 were purified by affinity chromatography by using *ProteIniso*® GST Resin. The size of GST tagfrom pGEX-4T-1 is about 26 kDa, and the protein sizes of LC1 and LC2 were about 27.1 kDa and 65.08 kDa, respectively; thereby, the recombinant protein sizes of rLC1 and rLC2 are about 53 kDa and 91 kDa, respectively. As shown in Figure 4, a single protein band of 45–60 kDa was observed in lane 2, and a single protein band of 80–100 kDa was observed in lane 3 in SDS-PAGE, and the results indicated that genes *lc1* and *lc2* were successfully expressed in *E. coli* BL21, and electrophoretic homogeneous rLC1 and rLC2 were obtained by *ProteIniso*® GST Resin.

2.2 Degradation activity of laccase, and the redox mediators assistance to rLC1

The preliminary test showed that 24 hours AFB1 degradation ratio of rLC1 was about 12% in degradation condition of this study, but degradation

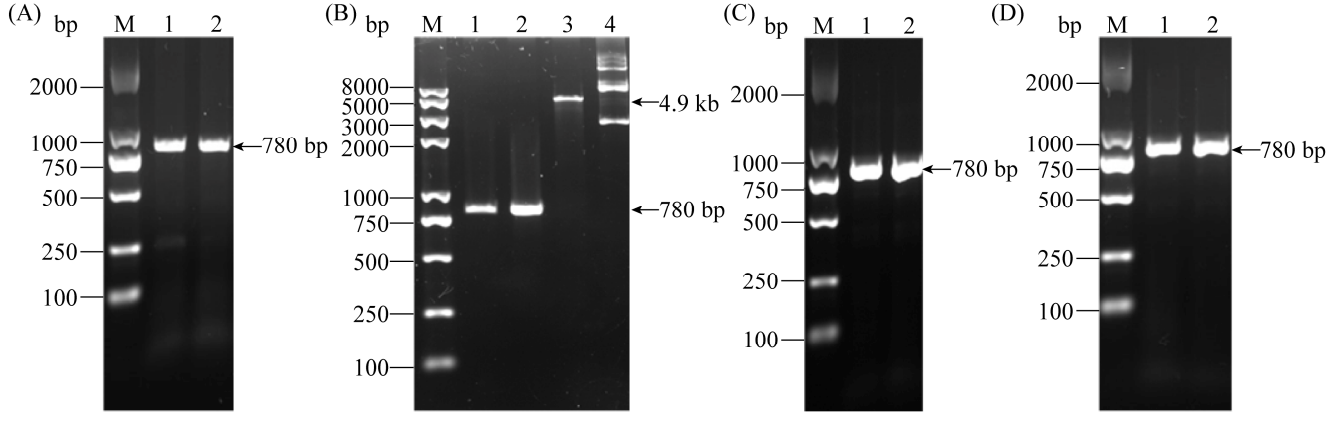

Figure 2. Gene cloning and heterologous expression (pGEX/*lc1*) of laccase gene *lc1* in strain *E. coli* BL21. A: the PCR fragment of gene *lc1*. B: *lc1* PCR product and pGEX plasmid double digestion (M: DNA marker; lane 1: *lc1* double digestion; lane 2: *lc1* PCR product; lane 3: pGEX-4T-1 double digestion products; lane 4: pGEX-4T-1). C: *lc1* PCR verification from recombinant plasmid pGEX/*lc1* in *E. coli* Trans1-T1. D: *lc1* PCR verification from recombinant plasmid pGEX/*lc1* in *E. coli* BL21.

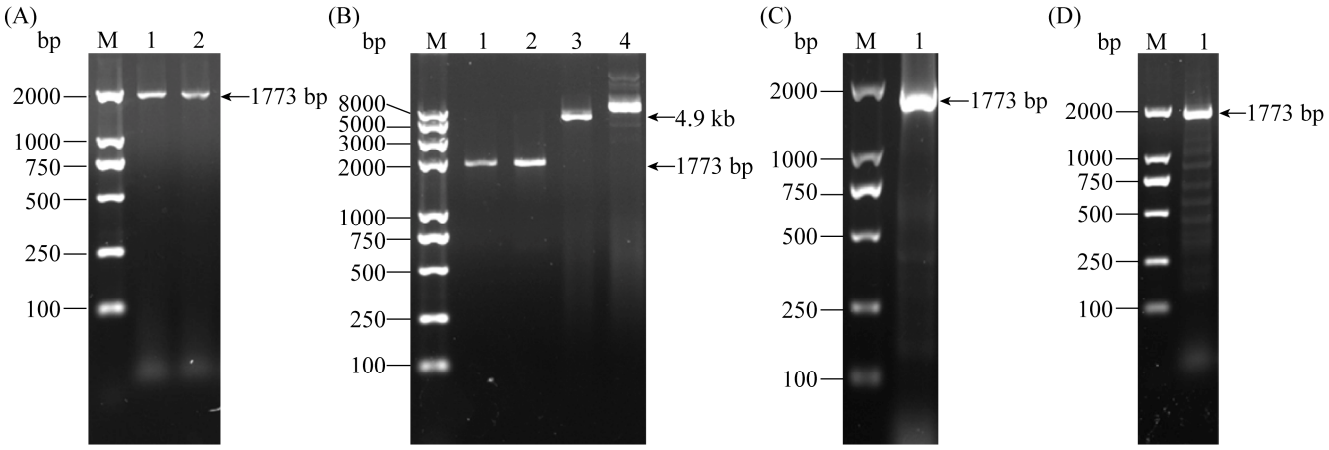

Figure 3. Gene cloning and heterologous expression (pGEX/*lc2*) of laccase gene *lc1* in strain *E. coli* BL21. A: the PCR fragment of gene *lc2*. B: *lc2* PCR product and pGEX plasmid double digestion. M: DNA marker; lane 1: *lc2* double digestion; lane 2: *lc2* PCR product; lane 3: pGEX-4T-1 double digestion; lane 4: pGEX-4T-1. C: *lc2* PCR verification from recombinant plasmid pGEX/*lc2* in *E. coli* Trans1-T1. D: *lc2* PCR verification from recombinant plasmid pGEX/*lc2* in *E. coli* BL21.

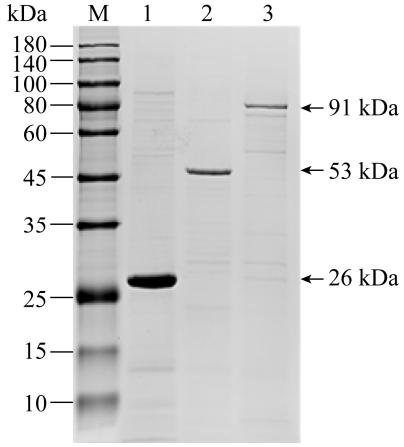

Figure 4. SDS-PAGE analysis of rLC1 and rLC2 purified protein. M: protein marker; lane 1: purified protein of GST tag; lane 2: purified protein rLC1; lane 3: purified protein rLC2.

activity was not observed on rLC2. As indicated by Loi *et al.*[21,23], redox mediators can enhance the AFB1 degradation activity of microbial laccases. In this study, the redox mediators of ABTS, AS and SA were tested for aflatoxin degradation assistance to rLCs. As a result, the three redox mediators of ABTS, AS and SA equal significantly enhanced the AFB1 degradation activity of rLC1; the most significant improvement was observed on ABTS as the additive, which improved the degradation ratio from 10.4% to 27.0% (Figure 5). However, the redox mediator ABTS also showed the AFB1 degradation activity (5.6%) which was not observed on the other two redox mediators (Figure 5).

2.3 Mutants CW117$^{\Delta lc1}$ and CW117$^{\Delta lc1\text{-}lc2}$ construction

By the PCR primer pairs of *lc1*-US and *lc1*-DS, the expected fragments of upstream (US, 1006 bp, Figure 6-A) and downstream (DS, 860 bp, Figure 6-B) flanking the gene *lc1* were successfully cloned. Then, the US and DS fragments were further lingated by overlap PCR with the primers of *lc1*-US-F and *lc1*-DS-R, and the overlap PCR product of *lc1*-US-DS fragment was 1886 bp. After the purification and sequencing validation, the purified PCR product *lc1*-US-DS was digested with *Hin*d Ⅲ and *Xba* Ⅰ, and ligated to digested suicide plasmid pK18*mobsacB*. The recombinant plasmid pK18*mobsacB*_*lc1*-US-DS was successfully constructed and validated in *E. coli* Trans1-T1 by PCR sequencing (Figure 6-C). After electroporation, the mutant CW117$^{\Delta lc1}$ was produced by 13% sucrose

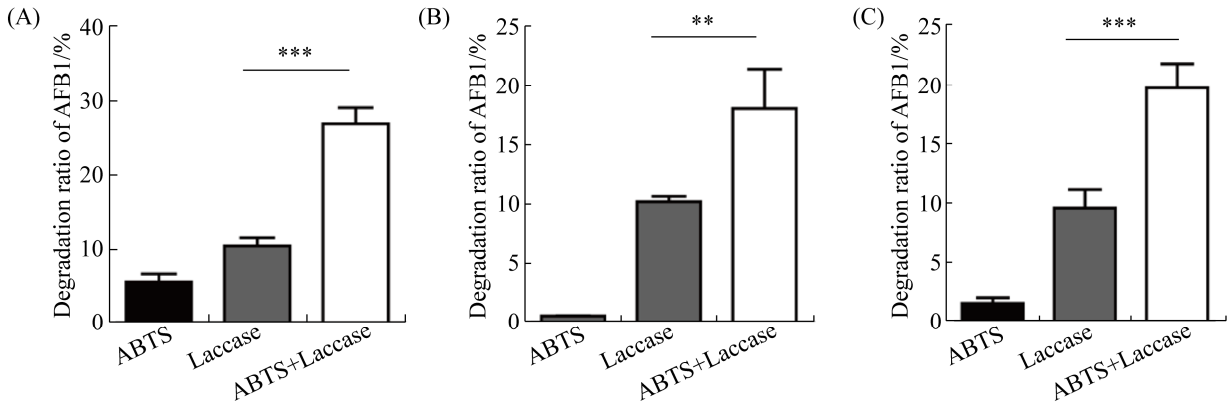

Figure 5. Aflatoxin B1 degrading activity of rLC1 supplemented with different redox mediators. A: the effect of ABTS. B: the effect of AS. C: the effect of SA. **: $P<0.01$, and ***: $P<0.001$, compared with the control (Laccase group).

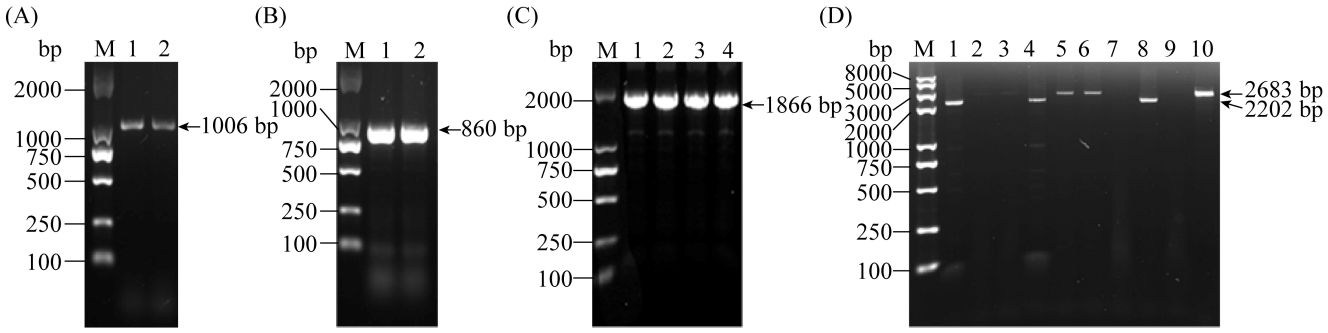

Figure 6. The gene *lc1* mutant construction from wide-type strain CW117. A: the upstream PCR fragment of gene *lc1*. B: the downstream PCR fragment of gene *lc1*. C: the PCR fragment of *lc1*-US-DS from pK18*mobsacB*_US-DS. D: the PCR validation on gene *lc1* mutant screening. M: DNA marker; lanes 1, 4, 8: positive candidates; lanes 5–6: negative colonies; lane 10: wild-type CW117.

and screened by Kan^R NA, and validated by PCR sequencing. The PCR fragment on the successful mutant $CW117^{\Delta lc1}$ was expected as 2202 bp, but the PCR fragment on wild-type CW117 was 2683 bp by using the same primer pair of ver1-F and ver1-R (Table 1). The screening results of this study showed that the band sizes (about 2.7 kb) of lanes 5, 6, 10 were consistent to wild-type CW117; while, the band sizes (about 2.2 kb) of lanes 1, 4 and 8 were considered as mutant $CW117^{\Delta lc1}$candidates (Figure 6-D). The screening results indicated that the monoclonal strains represented by lanes 5, 6, 10 probably restored to wild type, and the monoclonal strains represented by lanes 1, 4 and 8 might be the expected mutants. PCR sequencing results further validated the colonies from lanes 1, 4 and 8 was the positive mutant $CW117^{\Delta lc1}$.

By the PCR primer pairs of *lc2*-US and *lc2*-DS, we can see that the upstream and downstream fragments flanking the gene *lc2* were successfully amplified and connected together (Figure 7-A, B and C). The PCR fragment on the successful mutant $CW117^{\Delta lc1\text{-}lc2}$ was expected as 1637 bp, but the PCR fragment on starting strain $CW117^{\Delta lc1}$ without *lc2* deficiency was 3410 bp using the same primer pair of ver2-F and ver2-R (Table 1). The screening result

showed that the band size of lane 2 were consistent to starting strain CW117$^{\Delta lc1}$; while, the band size of lane 1 was considered as mutant CW117$^{\Delta lc1\text{-}lc2}$ candidate (Figure 7-D). PCR sequencing validated the clone from lane 1 was the positive mutant CW117$^{\Delta lc1\text{-}lc2}$.

2.4 The AFB1 degrading activity of wide-type and mutants

The degradation ratios of wild-type strain CW117 and the mutants (CW117$^{\Delta lc1}$ and CW117$^{\Delta lc1\text{-}lc2}$) were about 23% after 12 h incubation (no significant difference). After 24 h incubation, the degradation ratio of wide-type increased to 42.2% which a little higher than that of mutant CW117$^{\Delta lc1}$ (35.9%) and mutant CW117$^{\Delta lc1\text{-}lc2}$ (34.7%); however, the three strains (wide-type and two mutants) showed no significant difference from each other after 48 h incubation (Figure 8-A).

2.5 The AFB1 degradation activity of culture supernatant from wide-type and mutants

In previous study, we found that the AFB1 degradation agent(s) mainly distributed in culture supernatants (cell-free) the strain CW117[30]; thereby, the culture supernatants from the wide-type strain and

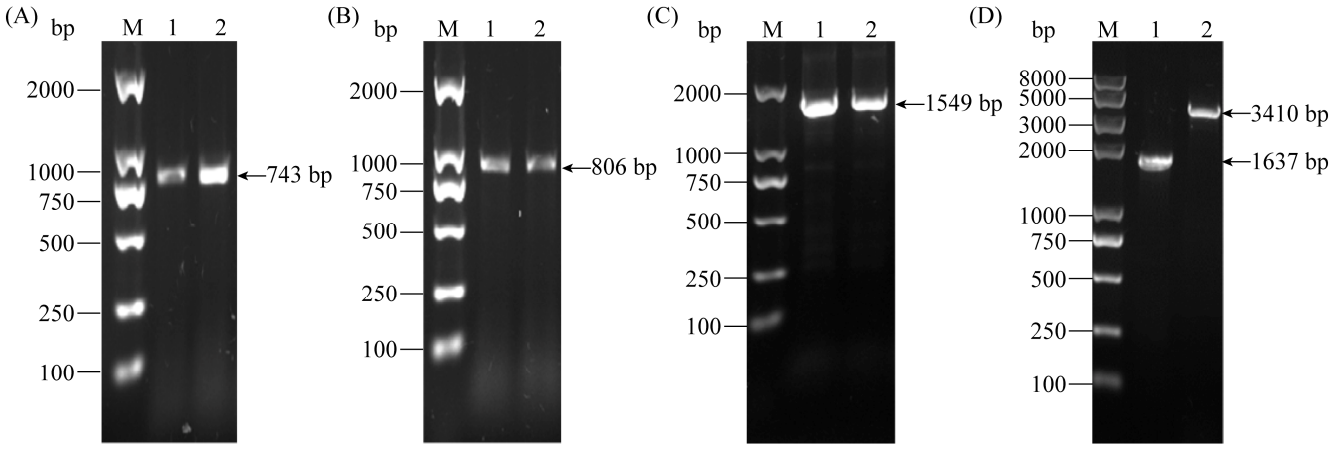

Figure 7. The genes *lc1-lc2* double mutant construction from staring strain CW117$^{\Delta lc1}$. A: the upstream PCR fragment of gene *lc2*. B: the downstream PCR fragment of gene *lc2*. C: the PCR fragment of *lc2*-US-DS from pK18*mobsacB*_US-DS. D: the PCR validation on gene *lc1-lc2* double mutant screening. M: DNA marker; lane 1: positive candidate; lane 2: staring strain CW117$^{\Delta lc1}$.

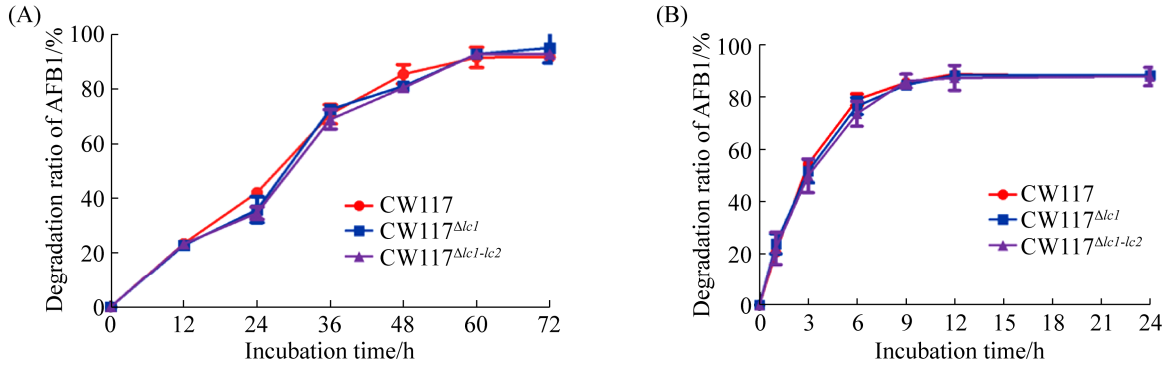

Figure 8. The AFB1 degradation dynamics of wide-type and mutants. A: the degradation dynamics of bacterial strains. B: the degradation dynamics of culture supernatant. Error bars indicate Mean±SD.

mutants were further evaluated here. Similar as the AFB1 degradation dynamics by bacterial strains (*i.e.*, CW117, CW117$^{\Delta lc1}$and CW117$^{\Delta lc1\text{-}lc2}$), the degradation activity by culture supernatants from wild type and mutants showed no significant difference from each other. The culture supernatants from three type strains (wide-type, *lc1* mutant and double genes mutant) degraded above 50% AFB1 within 3 h incubation, and degradation ratios increased to 80% within 9 h incubation (Figure 8-B).

3 Discussion

The degradation result of culture supernatant of CW117 was similar to the degradation characteristics of several previous studies, which equally showed the aflatoxin degrading agents in culture supernatant. For instance, the AFB1 degradation ratios by culture supernatant of *Bacillus shakletonii* L7[11], *Bacillus velezensis* DY3108[37], *Stenotrophomonas maltopholia* 35-3[18], *Mycobacterium* FA4[14] and *Rhodococcus erythropolis*[16] were 77.9%, 81.97%, 78.7%, 80% and 66.8% within 72 hours, respectively. The supernatants of *Candida versatilis* CGMCC 3790[38] and *Tetragenococcus halophilus* CGMCC 3792[39] also showed degradation activity to AFB1, the degradation ratios by the two strains' culture supernatant were 29.61% and 14% in 60 minutes, respectively. The similar degradation characteristics of the microbial strains might indicate the same degrading mechanism.

The redox mediators improvement on rLC1 of this study were much lower than the study of Lac2 from *Pleurotus pulmonarius*[21], which obtained an efficient degradation of AFB1 by adding 10 mmol/L AS (degradation ratio of 90%), SA (degradation ratio of 72%) and ABTS (degradation ratio of 81%) as redox mediators. Similarly, the additive improvements on rLC1 in this study were lower than the study of laccase Ery4 from *Pleurotus eryngii*[23], which obtained AFB1 degradation ratios of 73%, 68%, and 39% by adding 1 mmol/L AS, 1 mmol/L SA and 10 mmol/L ABTS, respectively. The original laccases of Lac2 and Ery4 also showed more efficient degradation than rLC1 of this study.

Substrate AFB1 in the culture medium could be degraded completely by any of the wide-type or mutant strain with increasing of incubation time. This result indicated that gene *lc1* showed degrading activity to AFB1, but the laccases (*i.e.*, *lc1* and *lc2*) could not be considered as the primary degradation agent in *Stenotrophomonas* sp. CW117.

The AFB1 degradation ratio of Lac2 purified from *Pleurotus pulmonarius* was 23% within 72 h, this degradation activity was similar to rLC1 of this study[21]. However, some other laccases, especially of laccases from several fungi strains showed more efficient aflatoxins degradation activity than rLC1. For instance, the laccase Ery4 from *Pleurotus eryngii* showed an AFB1 degradation ratio of 74% within 72 h[23]; the purified laccase (1 U/mL) from *Aspergillus niger* showed degradation ratio of 87.3% to AFB1 within 72 h[26]; the laccase from *Trametes versicolor* showed a degradation ratio of 67% to AFB1 within 48 h[29].

These studies suggested that laccases have been recognized as an effective aflatoxin degradation agent and widely distributed in microbial strains. For some degrading strains (*e.g.*, *Pleurotus pulmonarius* and *Stenotrophomonas* sp. of this study), the laccases degradation activity was not as efficient as their host strains. However, whether laccase(s) were the sole degradation agents in these degrading strains are not investigated further, the exact reasons for the degradation difference between laccase(s) and the host strains still unknown. In this study, when the laccase genes *lc1* and *lc2* were deleted, the mutant CW117$^{\Delta lc1}$and CW117$^{\Delta lc1\text{-}lc2}$ showed the similar degradation activity to wide-type strain CW117. The validation results of *lc1* and *lc2* degradation test *in vivo* indicated that laccases are not the sole degrading agents (even not the primary degrading agent) in *Stenotrophomonas* sp. CW117.

Degradation activity of culture supernatants were much higher than those of bacterial strains on the first 12 h. As we know, during the strains degradation test, first 12–24 hours are required for bacterial growth, but degrading agent(s) already existed in culture supernatants at the beginning of the test. The culture supernatant degradation tests equally showed that the laccase genes were not the sole (or even not the critical) degrading agent in *Stenotrophomonas* sp. CW117.

4 Conclusion

Laccases are the well-known aflatoxins degrading agent and widely distributed in microbial strains. In several previous studies, the aflatoxin degradation activities of laccases were not as efficient as the host strains, but the exact reasons were not investigated. In this study, two laccase genes (*lc1* and *lc2*) were isolated and evaluated for AFB1 degradation by heterologous expression. Compared to host strain CW117, laccase rLC1 showed much lower AFB1 degrading activity and rLC2 showed no activity on AFB1 transformation. The degradation results of mutants CW117$^{\Delta lc1}$and CW117$^{\Delta lc1\text{-}lc2}$ indicated that laccases are not the sole degrading agents (even not the critical factor) in *Stenotrophomonas* sp. CW117. And the finding of this study should be significant to further mechanism investigations on aflatoxins degradation.

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微嗜酸寡养单胞菌中的漆酶对黄曲霉毒素 B1 降解脱毒的生物活性

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摘要：【目的】探究微嗜酸寡养单胞菌中的漆酶对 AFB1 的降解活性，并确定漆酶在菌株 CW117 降解代谢 AFB1 过程中的贡献。【方法】从微嗜酸寡养单胞菌基因组中，共筛选到两个漆酶基因 *lc1* 和 *lc2*，并用大肠杆菌 BL21 外源表达蛋白 rLC1 和 rLC2，在体外检测其对 AFB1 的降解活性。同时参考前人报道，研究了氧化性辅剂对漆酶 AFB1 降解的促进作用。在体外实验基础上，利用自杀质粒 pK18*mobsacB*，以同源重组方法构建了两株漆酶缺失株 CW117$^{\Delta lc1}$ 和 CW117$^{\Delta lc1\text{-}lc2}$，验证了漆酶基因（*lc1* 和 *lc2*）对 AFB1 体内降解作用。【结果】体外实验显示，重组酶 rLC1 具有 AFB1 降解活性，氧化性辅剂 ABTS、AS 或 SA 可显著地提高 rLC1 降解活性，但 rLC2 未显示降解活性。突变株 CW117$^{\Delta lc1}$ 和 CW117$^{\Delta lc1\text{-}lc2}$ 对 AFB1 仍显示了较高的降解活性，且在大多数降解时间点与野生株 CW117 无显著差异。【结论】微嗜酸寡养单胞菌 CW117 菌株中，LC1 在体外显示了 AFB1 的降解活性，且降解活性可以被氧化性辅助因子增强，LC2 未显示体外降解活性；体内试验发现，漆酶基因 *lc1* 和 *lc2* 对菌株 CW117 降解 AFB1 的贡献较小，该菌株还存在其他降解途径。

关键词：解毒，漆酶，黄曲霉毒素 B1，降解基因，突变菌株

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