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Original research paper

Improved 11α-hydroxycanrenone production by modification of cytochrome P450 monooxygenase gene in *Aspergillus ochraceus*

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Eplerenone is a drug that protects the cardiovascular system. 11α-Hydroxycanrenone is a key intermediate in eplerenone synthesis. We found that although the cytochrome P450 (CYP) enzyme system in *Aspergillus ochraceus* strain MF018 could catalyse the conversion of canrenone to 11α-hydroxycanrenone, its biocatalytic efficiency is low. To improve the efficiency of 11α-hydroxycanrenone production, the CYP monooxygenase-coding gene of MF018 was predicted and cloned based on whole-genome sequencing results. A recombinant *A. ochraceus* strain MF010 with the high expression of CYP monooxygenase was then obtained through homologous recombination. The biocatalytic rate of this recombinant strain reached 93 % at 60 h without the addition of organic solvents or surfactants and was 17–18 % higher than that of the MF018 strain. Moreover, the biocatalytic time of the MF010 strain was reduced by more than 30 h compared with that of the MF018 strain. These results show that the recombinant *A. ochraceus* strain MF010 can overcome the limitation of substrate biocatalytic efficiency and thus holds a high potential for application in the industrial production of eplerenone.

Keywords: *Aspergillus ochraceus*, P450 monooxygenase, homologous recombination, canrenone, 11α-hydroxycanrenone

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Steroids are a group of compounds that contain a four-ring structure, termed as the cyclopentanoperhydrophenanthrene nucleus, and possess a wide range of pharmacological activities (1–3). Although natural steroids tend to have low pharmacological activity, their pharmacological and biological activities can be drastically enhanced by introducing specific chemical groups at specific sites (1, 4, 5). These sites, such as C11α, C11β, C9α and C16α, are important hydroxylation sites. C11α-hydroxylation is one of the most important reactions (2, 6) because the modification of C11α, a physiologically active group, can greatly increase therapeutic effects, reduce side effects and change the specificity of steroids (7).

Canrenone, a cardiovascular drug, is a steroid hormone that is used primarily as a nonselective aldosterone receptor antagonist in clinical medicine. It treats cardiovascular

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diseases by blocking the renin-angiotensin-aldosterone system (8, 9). However, canrenone has a negative interference effect on the cardiotonic compound digoxin; thus, the use of canrenone in heart disease treatment may cause dangerous side effects or death (8, 10). The derivatives of canrenone are developed *via* nitration, hydroxylation and other modifications to enhance its efficacy and reduce side effects (11). Amongst these methods, the 11α-hydroxylation of canrenone by microorganisms to produce 11α-hydroxycanrenone is a promising strategy for producing modified canrenone derivatives.

Eplerenone is a new selective steroidal aldosterone receptor antagonist. Clinical studies have demonstrated that in cardiovascular diseases, such as hypertension, the side effects and mortality associated with heart failure disorders are significantly reduced by eplerenone treatment compared with those by canrenone treatment (12–14). The chemical synthesis of eplerenone is a six-step process with 11α-hydroxycanrenone as an intermediate (15). Microbial catalytic conversion from canrenone to 11α-hydroxycanrenone is the first and the most critical step during eplerenone synthesis (16, 17).

The 11α-hydroxylation of steroids in microorganisms is associated with the intracellular enzyme 11α-hydroxylase, which belongs to the cytochrome P450 (CYP)-dependent monooxygenase family (18, 19). This enzyme introduces oxygen atoms at specific sites in the steroid backbone to convert lipophilic steroidal compounds into relatively hydrophilic derivatives (18). Cytochrome C reductase (CPR) and hexane oxidase are mediated by CYP in *Aspergillus ochraceus*, and these CYP-linked monooxygenases can be used to catalyse the conversion of cycloxanthone into 11α-hydroxyxanthone (19, 20). However, the biocatalytic specificities of *A. ochraceus* vary for different steroid substrates, and its catalytic efficiency is low. Increasing the catalytic efficiency of *A. ochraceus* for steroids involves two aspects: the optimisation of fermentation conditions and the genetic modification of strains. To date, many studies have focused on the optimisation of the fermentation conditions of steroids produced by *A. ochraceus*, but few have focused on the genetic modification of *A. ochraceus*. The genetic modification approach can help resolve key technical difficulties and improve the efficiency of steroid catalysis.

An *A. ochraceus* strain MF018 with the ability to 11α-hydroxylate steroids was isolated in our laboratory, but its biocatalytic efficiency was found to be low. To improve the efficiency, a recombinant *A. ochraceus* strain with high expression of the CYP monooxygenase gene under the control of the *Tr* promoter was constructed *via* homologous recombination. This strain could improve the biocatalytic efficiency of the 11α-hydroxylation of steroids and exhibits potential industrial applications.

EXPERIMENTAL

Strain and culture conditions

Escherichia coli DH5α and *A. ochraceus* MF018 strains were preserved in the laboratory. A single colony of *E. coli* DH5α was inoculated in Luria-Bertani medium (21) with 100 μg mL^{-1} ampicillin and cultured at 37 °C and 200 rpm overnight.

The spores of *A. ochraceus* were collected on potato dextrose agar (PDA) medium (22, 23) and cultured in seed medium (6 g L^{-1} glucose, 4 g L^{-1} soluble starch, 20 g L^{-1} corn syrup, 10 g L^{-1} peptone, 1 g L^{-1} $NH_4H_2PO_4$, 2 g L^{-1} $MgCl_2$, pH 6.4) at 28 °C and 180 rpm for 18 h.

The culture was transferred to fermentation medium (25 g L^{-1} glucose, 10 g L^{-1} soluble starch, 20 g L^{-1} corn syrup, 10 g L^{-1} peptone, 1 g L^{-1} $NH_4H_2PO_4$, 2 g L^{-1} $MgCl_2$, natural pH) with an inoculum size of 75 mg mycelium dry weight per litre and was cultured at 28 °C and 200 rpm.

Whole-genome sequencing of A. ochraceus *and prediction of the CYP monooxygenase gene*

The genomic DNA of *A. ochraceus* MF018 was extracted using an OMEGA-DNA extraction kit and sent to Suzhou GENEWIZ for whole-genome sequencing. The genomic sequence of *A. ochraceus* MF018 was determined using PacBio sequel systems powered by single-molecule real-time (SMRT) sequencing technology. The genome was assembled using Falcon. The assembly results were corrected by using the calibration software Quiver (version 1.1.0). For gene prediction, the start sites and coding regions were effectively predicted using the software Augustus (version 3.3). Noncoding RNAs were obtained through sequence alignment with the Rfam database (version 12.0). Target sequences were searched using blast-2.2.30+111 following the CYP monooxygenase gene (GenBank No. ABH71415.1).

Antibiotic resistance screening of A. ochraceus *MF018*

The spores of *A. ochraceus* MF018 were collected from PDA medium and resuspended in sterile 0.9 % NaCl solution at a concentration of 10^7–10^8 mL^{-1}. Next, 100 μL of spore suspension was applied to PDA medium containing hygromycin B or bleomycin (Blm) at final concentrations of 50, 75, 100, 125, 150 and 200 μg mL^{-1} for each antibiotic. Cells were cultured at 28 °C for 4–6 days. The experiments were repeated three times.

Gene cloning and construction of the recombinant plasmid

The characteristics of primers and plasmids used in this study are shown in Tables I and II, respectively. The strategy for fusion gene construction is shown in Fig. 1a.

The polymerase chain reaction (PCR) primer pairs (Table I) were designed based on the predicted CYP monooxygenase gene (*cypm*). The PCR amplification parameters were 1 cycle of denaturation (95 °C for 5 min), 30 cycles of extension (95 °C for 45 s, 53 °C for 30 s and 72 °C for 1 min 35 s) and 1 cycle of annealing (72 °C for 10 min). The *cypm* gene was cloned using the genomic DNA of *A. ochraceus* MF018 as a template. The cloned gene was inserted into the vector pMD-18T (Table II) to construct the plasmid pMD18-Cyp.

The upstream homologous arm sequence of *cypm* (Up) was amplified through PCR using F-Up/R-Up as the primer pair and *A. ochraceus* MF018 genomic DNA as the template. Then, the plasmid pMD18-Up was constructed. Similarly, the downstream homologous arm sequence of *cypm* (Down) was amplified by applying the same PCR protocol using primer pair F-Down/R-Down and *A. ochraceus* MF018 genomic DNA as a template. Then, the plasmid pMD18-Down was constructed. The promoter sequence (*Tr*) was amplified by using F-Tr/R-Tr as the primer pair and pSilent as the template to construct the plasmid pMD18-Tr. The hygromycin resistance gene (*hyg*) was amplified using F-Tr/R-Hyg as the primer pair and the plasmid pAg1-H3 as the template to construct the plasmid pMD18-Hyg.

Table I. Primer sequences for construction of recombinant strains and description

Name	Sequence (5′–3′)	Description
F-Cyp	ATGCCCTTCTTCACTGGGCTTCT	*cypm*[a]
R-Cyp	CTACACAGTTAAACTCGCCA	
F-Tr	CTGATATTGAAGGAGCAC	*Tr* promoter sequence
R-Tr	ATCGATGCTTGGGTAGAATAGG	
F-Down	ACTCTAACTGCCGATGGAG	Downstream homology arm sequence of *cypm*
R-Down	ATGCCCTTCTTCACTGGGCTTCT	
F-Up	GGTGATACAATGATTCGGAGA	Upstream homology arm sequence of *cypm*
R-Up	GATAATGAGCTGTCAGCTTGTGG	
F-Tr	CTGATATTGAAGGAGCAC	Hygromycin resistance gene
R-Hyg	TAGTTCTAGAGCGGCCGCAA	
F-Link1	GCTGACAGCTCATTATCCTGATATTGAAGGAGCAC	Fusion fragment UT[b]
R-Link1	GTGCTCCTTCAATATCAGGATAATGAGCTGTCAGC	
F-Link2	TCTACCCAAGCATCGATATGCCCTTCTTCACTGG	Fusion fragment UTP[c]
R-Link2	CCAGTGAAGAAGGGCATATCGATGCTTGGGTAGA	
F-Link3	TTGCGGCCGCTCTAGAACTATAGGTCGAACACGAAGTCC	Fusion fragment HD[d]
R-Link3	GGACTTCGTGTTCGACCTATAGTTCTAGAGCGGCCGCAA	
F-Link4	GGCGAGTTTAACTGTGTAGCTGATATTGAAGGAGCAC	Complete fusion fragment UTPHD[e]
R-Link4	GTGCTCCTTCAATATCAGCTACACAGTTAAACTCGCC	
F-Su	ACTAGTGGTGATACAATGATTCGGAGA	
R-Ad	GGGCCCACTCTAACTGCCGATGGAG	
F-Screen	CCAGTCAACCTTCTCGGTCG	Verification of the recombinant strain
R-Screen	GCTGGCAGAGTGCACTTCG	

[a] Cytochrome P450 monooxygenase gene.
[b] The fusion fragment of upstream homologous arm sequence of *cypm* and *Tr*.
[c] The fusion fragment of the UT and *cypm*.
[d] The fusion fragment of hygromycin resistance gene and downstream homologous arm sequence of *cypm*.
[e] The complete fusion gene fragment of the UTP and HD.

Fusion PCR was performed to ligate the Up, *Tr*, *cypm*, *hyg* and Down fragments together (Fig. 1a). The constructed complete fusion fragment (UTPHD) was inserted into the plasmid pB-Hygro to construct the recombinant plasmid. The method is described in detail below:

First step: The upstream homologous arm fusion fragment (U-L1R) was amplified using the primer pair F-Up/R-link1 and pMD18-Up as the template. The promoter fusion fragment (T-L1F) was amplified using the primer pair F-link1/R-Tr and pMD18-Tr as the

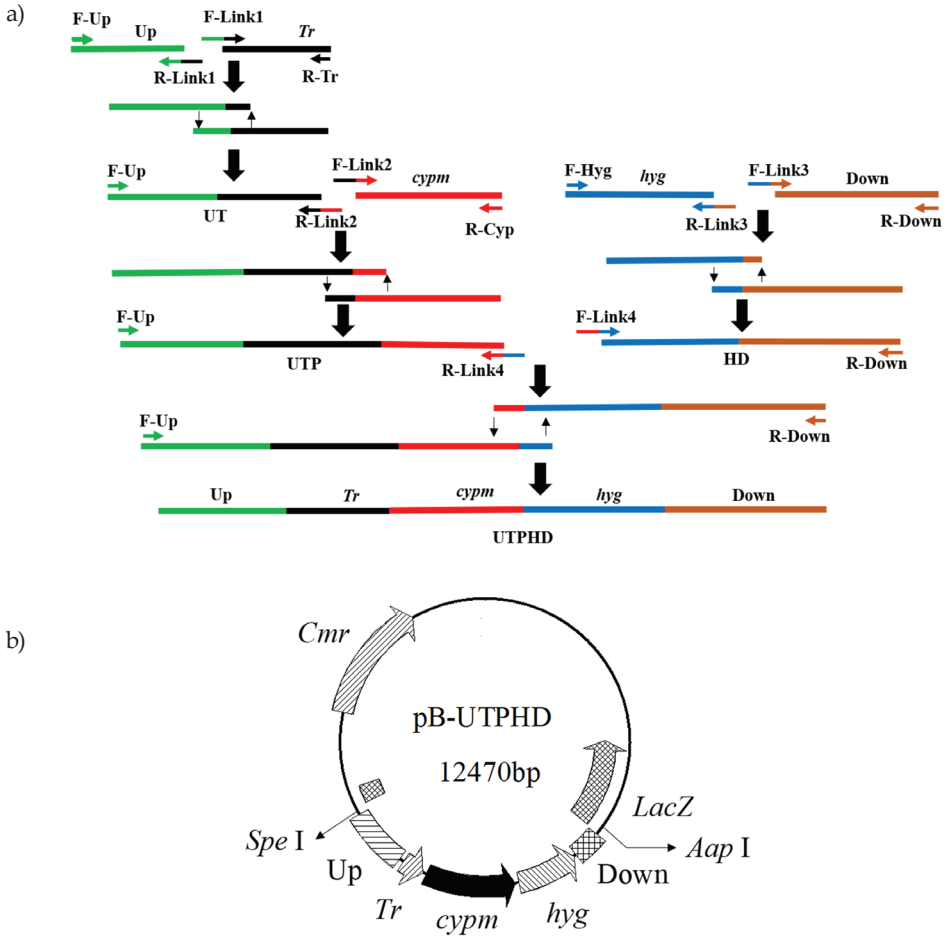

Fig. 1. Profile of: a) construction principle of fusion genes and b) the recombinant plasmid pB-UTPHD.

template. The PCR products were detected through gel electrophoresis, and the target bands were purified by gel recovery (SanPrep Column DNA Gel Extraction Kit).

Second step: The PCR amplification was performed using 0.5 μL of TaKaRa LA Taq (5 U μL^{-1}), 2.5 μL of 10× LA Taq Buffer II (Mg^{2+} Plus), 4 μL of dNTP Mixture (2.5 mM each), 1 μL of template U-L1R (< 1 μg), 1 μL of template T-L1F (< 1 μg) and 16 μL of H_2O. Primers F-Up and R-Tr were added after 10 cycles of amplification in accordance to the procedure described above, and amplification was performed for 20 cycles. The upstream homologous arm of *cypm* and the promoter sequence fusion fragment (UT) were purified, and the recombinant plasmid pMD18-UT was constructed.

Third step: The plasmids pMD18-UT and pMD18-Cyp were used as templates to amplify the fusion fragment (UTP) with the upstream homologous arm of *cypm*, the pro-

Table II. Plasmids and the description

Plasmids	Description	Source
pMD18-T	Ampicillin resistance gene (*Ampr*)	Takara Co. Ltd.
pB-Hygro	*E. coli/A. ochraceus* shuttle vector, chloramphenicol resistance gene	Fenghui Bio Co. Ltd.
pSilent-Dual	For amplification of *Tr* promoter sequence, *Ampr*	Fenghui Bio Co. Ltd.
pAgI-H3	For amplification of hygromycin resistance gene, kanamycin resistance gene	Miao Ling Plasmid Co. Ltd.
pMD18-Cyp	Containing the *cypm*[a], *Ampr*	This research
pMD18-Tr	Containing *Tr* promoter sequence, *Ampr*	This research
pMD18-Up	Containing upstream homologous arm sequence of *cypm*, *Ampr*	This research
pMD18-Down	Containing downstream homologous arm sequence of *cypm*, *Ampr*	This research
pMD18-Hyg	Containing hygromycin resistance gene, *Ampr*	This research
pMD18-UT	Containing the fusion fragment UT[b], *Ampr*	This research
pMD18-UTP	Containing the fusion fragment UTP[c], *Ampr*	This research
pMD18-HD	Containing the fusion fragment HD[d], *Ampr*	This research
pMD18-UTPHD	Containing the complete fusion fragment UTPHD[e], *Ampr*	This research
pB-UTPHD	Recombinant plasmid, *hyg*	This research

[a] Cytochrome P450 monooxygenase gene.
[b] The fusion fragment of upstream homologous arm sequence of *cypm* and *Tr*.
[c] The fusion fragment of the UT and *cypm*.
[d] The fusion fragment of hygromycin resistance gene and downstream homologous arm sequence of *cypm*.
[e] The complete fusion gene fragment of the UTP and HD.

moter sequence and *cypm* using the same strategy as that described in the second step. The primer pairs F-Up/R-link2, F-link2/R-Cyp and F-Up/R-Cyp were used with both templates.

Fourth step: The plasmids pMD18-Hyg and pMD18-Down were used as templates to amplify the fusion fragment (HD) with the *hyg* gene and the downstream homologous arm of *cypm* using the same strategy as that described in the second step. The primer pairs F-Tr/R-link3, F-link3/R-Down and F-Tr/R-Down were used.

Fifth step: The plasmids pMD18-UTP and pMD18-HD were used as templates to amplify the UTPHD with fusion fragments UTP and HD using the same strategy as that described in the second step. The primer pairs F-Up/R-link4, F-link4/R-Down and F-Su/R-Ad were used.

The plasmid pMD18-UTPHD was digested with the restriction enzymes *Aap* I and *Spe* I. The fusion gene fragment UTPHD was then purified and inserted into the pB-hygro plasmid digested with the same restriction enzymes, and the recombinant plasmid desig-

nated pB-UTPHD was constructed (Fig. 1b). The plasmid pB-UTPHD was then transformed into *E. coli* DH5α competent cells (24) and screened for chloramphenicol resistance (20 μg mL^{-1}). Positive transformants were verified through PCR using the primer pairs F-Down/R-Ad, F-Tr/R-Hyg, F-Cyp/R-Cyp and F-Su/R-Tr. Then, the recombinant plasmid pB-UTPHD was sequenced to confirm the absence of any mutation generated by PCR.

Preparation of A. ochraceus protoplasts and transformation

The protoplasts of *A. ochraceus* MF018 were prepared, and the plasmid pB-UTPHD was transformed as described by Weyda *et al.* (25, 26). The protoplasts were cultured for 6–8 h in RM medium (1 g L^{-1} casein amino acid, 1 g L^{-1} yeast extract, 274 g L^{-1} sucrose). The cells were applied to the PDA medium containing 125 μg mL^{-1} hygromycin B and cultured at 28 °C for 5–6 days. Positive transformants were propagated for 6–7 generations to assess their stability before being verified by PCR amplification using the primers F-Screen and R-Screen (Table I). *A. ochraceus* MF018 was used as the negative control.

Growth curve determination

The constructed *A. ochraceus* MF010 was cultured as described above. A total of 50 mL of culture was taken at 5-h intervals and filtered. The mycelia were then dried to a constant weight. *A. ochraceus* MF018 was used as negative control.

Determination of conversion products by HPLC-MS

A. ochraceus MF010 was cultured as described above. Canrenone was added after 18 h of culture at a final concentration of 20 g L^{-1}. After 24 h of incubation at 28 °C and 200 rpm, the sample was extracted with 2 volumes of ethyl acetate. The supernatant was then diluted 50 times with methanol and analysed by HPLC-MS. The chromatographic conditions applied: a mobile phase methanol/water (8:2, *V*/*V*), the injection volume 10 μL, the flow rate of 0.8 mL min^{-1}. Mass spectrometry analysis was performed as described by Huang *et al.* (17, 27).

Determination of canrenone and 11α-hydroxycanrenone by HPLC

A. ochraceus MF010 was cultured as described above. The concentration of canrenone substrate was determined at every 12-h interval. The canrenone conversion rate was calculated using Equation *1*:

$$\text{Molar conversion rate}\% = \frac{340.46X}{356.46Y} \times 100\% \qquad (1)$$

where *X* and *Y* are 11α-hydroxycanrenone (g L^{-1}) and canrenone (g L^{-1}), respectively. *A. ochraceus* MF018 was used as negative control.

Data analysis

Statistical analysis was performed using OriginPro 2016. All quantitative data are expressed as the mean ± standard deviation for each condition.

RESULTS AND DISCUSSION

Annotation of the A. ochraceus *genome and prediction of the cypm*

The genomic sequence of the *A. ochraceus* strain MF018 was determined by using PacBio's SMRT sequencing technology with a highly accurate sequencing of over 99.999 % (QV50) independent of the GC and AT content of DNA sequences. The draft genome of *A. ochraceus* MF018 consisted of 164 sequence contigs with a total length of 3,709,426 bp and an N_{50} value of 1,732,139 bp. The average contig length was 226,184.52 bp, and the G+C content was 48.74 %. The numbers of predicted protein-coding genes and ncRNAs were 10,535 and 1235, respectively. The average protein-coding gene density was 1 gene per 1638.09 bp. The protein-coding sequences occupied 46.5 % of the sequenced portion of the *A. ochraceus* MF018 genome. Genomic information has been published in GenBank (GenBank No. VBTP00000000).

The *cypm* gene, which has a total length of 1834 bp, was retrieved from the sequencing assembly result of *A. ochrace*us MF018 using the software blast-2.2.30+111. Its similarity with the previously reported CYP monooxygenase gene (GenBank: ABH71415.1) was 99 % (28). Sequence alignment revealed that the *cypm* gene of *A. ochraceus* MF018 contained four introns with a coding sequence length of 1542 bp.

Antibiotic resistance gene selection

The antibiotic resistance gene *hyg* was selected as a screening marker for the recombinant strain. The antibiotic resistance of the unmodified *A. ochraceus* strain MF018 was determined first. The number of *A. ochraceus* MF018 colony-forming units was 0 when the hygromycin B concentration exceeded 100 μg mL^{-1} and the concentration of Blm reached 125 μg mL^{-1}. The growth inhibition of *A. ochraceus* MF018 by hygromycin B was higher than that by Blm at the same concentration. Hygromycin B was used at a final concentration of 125 μg mL^{-1} to reduce the impact of false-positive strains during the construction of the recombinant strain.

Construction of the recombinant plasmid pB-UTPHD

An 1834-bp sequence of the *cypm* gene was obtained using the genomic DNA of *A. ochraceus* MF018 as a template (Fig. 2a). The sequences of Up, Down, *Tr* and *hyg* were 1011, 610, 359 and 1908 bp, respectively. Using these DNA fragments, the sequences of the fusion gene fragments UT, UTP and HD were 1370, 3204 and 2518 bp, respectively. The sequence of the fusion fragment UTPHD was 5722 bp. The profile of the recombinant plasmid pB-UTPHD and the PCR results are shown in Fig. 1b and Fig. 2b, respectively. The sequencing results were consistent with the predictions, and the successful construction of the recombinant plasmid pB-UTPHD was confirmed.

Construction of recombinant A. ochraceus *MF010*

The recombinant plasmid pB-UTPHD was transferred into the *A. ochraceus* MF018 recipient strain as described by Yang and Weyda *et al.* (25, 26). To confirm the double crossover at the *cypm* position in the chromosome of *A. ochraceus* MF018, a 6016-bp gene

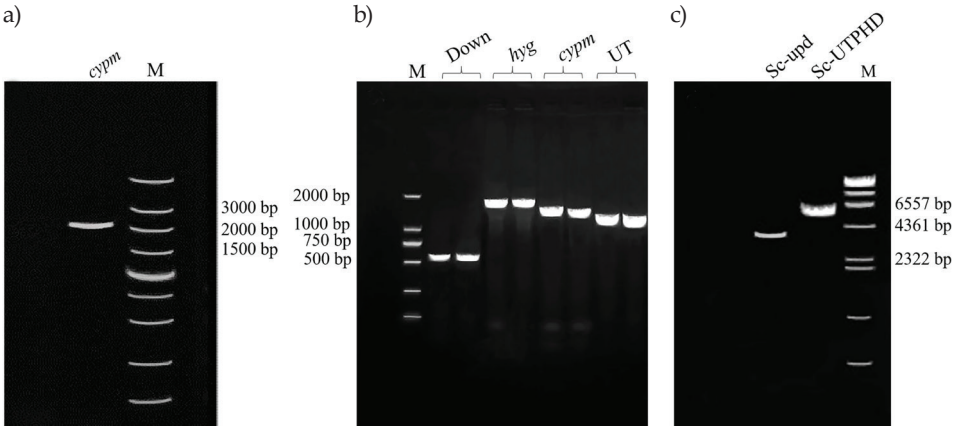

Fig. 2. Agarose gel electrophoresis analysis of PCR products of: a) the *cypm* and b) the recombinant plasmid pB-UTPHD; c) the fragments amplified from the genomic DNA of *A. ochraceus* MF018 and MF010. *cypm*, cytochrome P450 monooxygenase gene; Down, Downstream homology arm sequence of *cypm*; *hyg*, hygromycin resistance gene; UT, the fusion fragment of upstream homologous arm sequence of *cypm* and *Tr* promoter sequence; Sc-upd, PCR products amplified from the MF018 genomic DNA template; Sc-UTPHD, PCR products amplified from the MF010 genomic DNA template.

fragment (designated as Sc-UTPHD) was amplified through PCR by using the primer pair F-Screen/R-Screen (Fig. 2c). Additionally, a 3750-bp gene fragment (designated as Sc-upd) was amplified from *A. ochraceus* MF018 as the negative control by using the same primers (Fig. 2c). In summary, *A. ochraceus* MF010 with the desired *cypm* mutation was constructed through homologous recombination by double crossover.

Growth curve of A. ochraceus *MF010*

The growth curve of *A. ochraceus* MF010 strain was determined to determine the influence of homologous recombination with the modified *cypm* on the growth of this strain. *A. ochraceus* MF018 was used as the control. The growth retardation period, logarithmic growth phase, stationary growth period and decline period were 0–8, 8–31, 31–50 and 50 h, respectively. The growth curve of *A. ochraceus* MF010 was almost the same as that of *A. ochraceus* MF018. *A. ochraceus* MF010 showed good growth conditions, strong cell viability and normal basal metabolism.

Biocatalytic conversion of canrenone to 11α-hydroxycanrenone by A. ochraceus *MF010*

After culturing, the product of *A. ochraceus* MF010 was analysed through HPLC-MS. The molecular weight of the canrenone substrate was 341.29 after adding a hydrogen atom and 363.26 after adding a sodium atom (Fig. 3a). Therefore, the molecular weight of the canrenone substrate was approximately 340.27. The molecular weight of the biocatalytic product was 357.27 after adding a hydrogen atom and 379.25 after adding a sodium atom (Fig. 3b). Thus, the molecular weight of the product was approximately 356.26, *i.e.*

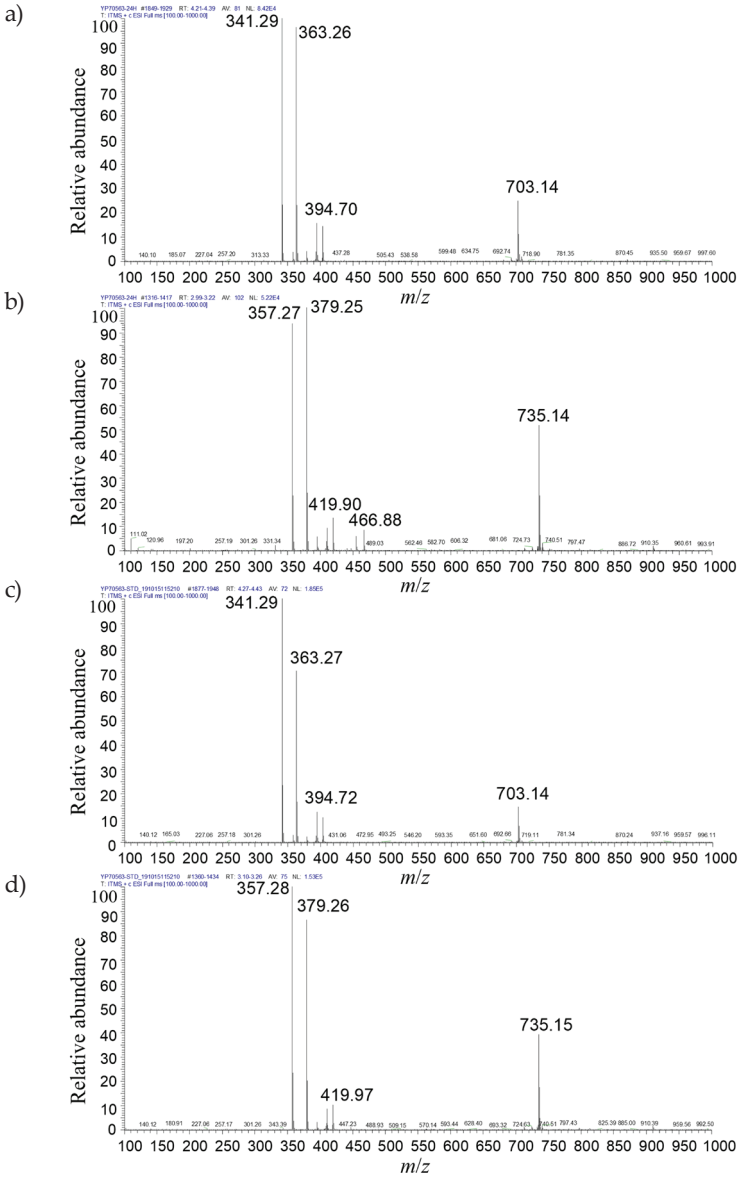

Fig. 3. HPLC-MS spectra of: a) canrenone in culture after biocatalysis, b) 11α-hydroxycanrenone in culture after biocatalysis, c) 0.2 g L^{-1} of canrenone standard and d) 0.2 g L^{-1} of 11α-hydroxycanrenone standard.

approximately 16 more than that of the canrenone substrate. The data were consistent with those reported previously (17, 27, 29) and the standards (Fig. 3c and 3d). These data demonstrated that *A. ochraceus* strain MF010 catalyses canrenone to 11α-hydroxycanrenone.

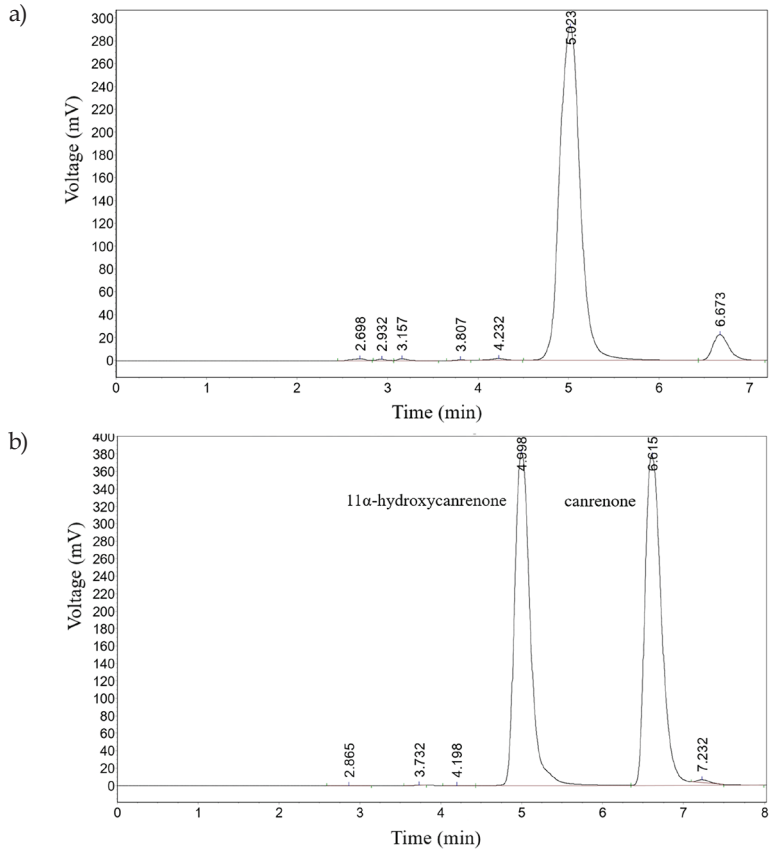

Fig. 4. HPLC spectra of: a) the main products of *A. ochraceus* MF010 after biocatalysis, b) standards of canrenone and 11α-hydroxycanrenone.

Additionally, the HPLC results showed that the retention times of the product and substrate were 5.023 and 6.673 min, respectively (Fig. 4a). The results were consistent with the standards of 11α-hydroxycanrenone and canrenone (Fig. 4b).

Further, the substrate conversion rate was calculated on the basis of the peak area. When canrenone was added at a final concentration of 20 g L^{-1}, the substrate biocatalytic conversion rate of *A. ochraceus* MF010 exceeded that of *A. ochraceus* MF018 (Fig. 5). Without the addition of any organic solvent or surfactant, the substrate conversion rate of *A. ochraceus* MF010 reached 93 % at 60 h, whereas that of *A. ochraceus* MF018 reached 75 % at 96 h. The highest conversion rate of *A. ochraceus* MF010 was 17–18 % higher than that of *A. ochraceus* MF018. The biocatalytic time of *A. ochraceus* MF010 was also reduced by more than 30 h compared with that of *A. ochraceus* MF018. These results indicated that the conversion rate of *A. ochraceus* MF010 was superior to that of *A. ochraceus* MF018 with a significantly shorter biocatalytic time.

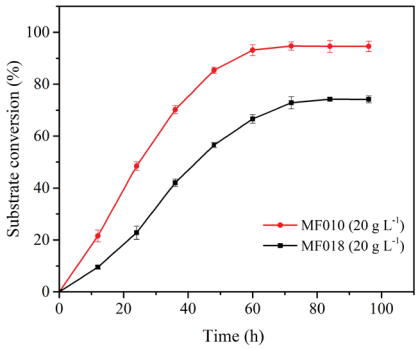

Fig. 5. Time courses of canrenone conversion rates of *A. ochraceus* strains MF010 and MF018.

Many recent studies have focused on improving the biocatalytic efficiency in terms of cell immobilisation or selection and the optimisation of fermentation conditions. Houng *et al*. (30) reported a novel cell immobilisation technique that was developed for increasing substrate partitioning to the gel matrix by coating a thin polyurea layer on the surfaces of calcium alginate beads. This method improved the efficiency of the conversion of progesterone to 11α-hydroxyprogesterone by *A. ochraceus*. Rong *et al*. (31) reported a new method for the biocatalytic conversion of β-sitosterol to 11α-hydroxyandrosta-1,4-diene-3,17-dione (11α-OH-ADD) through the cultivation of a *Mycobacterium* MF006 and *A. ochraceus* MF007 mixture with hollow organic silica spheres. This method improved the dispersibility of the substrate based on its excellent adsorption properties, thereby improving the conversion efficiency of β-sitosterol to 11α-OH-ADD. These studies focused on improving the efficiency of the catalytic process of *A. ochraceus* but did not change the genetic characteristics of the strain. Wang *et al*. (32) identified a novel 11α-hydroxylase gene *CYP5311B1* from *Absidia coerulea* AS3.65 through heterologous expression in *Pichia pastoris*. Its expression at the transcriptional level could be highly induced by steroid substrates. Another 11α-hydroxylase gene called *CYP509C12* from *Rhizopus oryzae* was cloned and identified, and its heterologous expression and functional identification were performed in fission yeast (33). Hull *et al*. (34) reported that the genes encoding 11α-steroid hydroxylase enzymes from *A. ochraceus* and *R. oryzae* could be transformed into *Saccharomyces cerevisiae* for heterologous constitutive expression and that both recombinant yeasts exhibited the efficient bioconversion of progesterone.

The electrons required for the catalytic activity of the fungal steroid hydroxylase are provided by NAD(P)H-CYP reductase. Electron transfer to CYP can be a rate-limiting step in various P450 systems (35–37). Most research on the cloning and expression of *cypm* and 11α-hydroxylation of steroids was performed using heterologous expression systems, such as yeast, which cannot efficiently support hydroxylation. Petrič *et al*. (33) attempted to clone CYP and coexpress it with its natural redox partner (CPR) from *Rhizopus oryzae* in a fission yeast strain under inducible conditions. Compared with that by the fission yeast strain expressing only CYP, the total yield of hydroxyprogesterone by fission yeast strain coexpressing CYP and CPR increased by approximately 20 % with a 7-fold improvement in the bioconversion rate at 24 h. Additionally, factors such as codon preference can limit the heterologous expression efficiency of 11α-hydroxylase. The use of *A. ochraceus* as an

expression system could effectively solve these problems. *A. ochraceus* grows quickly and is easy to culture. It also provides advantages in the expression and secretion of proteins (38). Yang *et al*. (25) reported a recombinant strain of *A. ochraceus* TCCC41060 that could overexpress the steroid 11α-hydroxylase. Its transformation time was significantly reduced compared with that of the wild strain. After 48 h of conversion, the recombinant strain maintained a high 16,17a-epoxyprogesterone (EP) conversion rate (approximately 75 %) when EP concentrations were 5 g L^{-1}. However, compared with the high-yielding *A. ochraceus* strain, a gap remains in the conversion rate. When 1.5 g L^{-1} of the surfactant Tween-80 was added, the conversion rate of the recombinant strain could increase to 92 % at 48 h. However, studies have demonstrated that the addition of nonionic surfactants could delay spore germination, inhibit cell growth, lead to membrane damage and result in cellular metabolite leakage. The use of excessive concentrations of organic solvents might cause mycelial breakage or even autolysis (39). Additionally, the use of a cosolvent might cause problems during product extraction, separation and purification; these problems might also increase the production cost.

In this study, the *A. ochraceus* strain MF010 exhibiting high expression of CYP monooxygenase was constructed through the homologous recombination of promoter *Tr* and CYP on the chromosome. The biocatalytic rate of *A. ochraceus* MF010 reached approximately 93 % at 60 h without the addition of any organic solvent or surfactant. The original *A. ochraceus* strain MF018 only achieved a biocatalytic rate of approximately 75 % at 96 h under the same conditions. The genetic stability of this expression system was higher than that of heterologous expression systems, such as yeast. It can also efficiently provide the electrons required for the catalytic activity of the fungal steroid hydroxylase Furthermore, it effectively avoided the damage caused by organic solvents and surfactants and was beneficial for the separation and purification of 11α-hydroxycanrenone. The recombinant strain overcame the limitation of substrate biocatalytic efficiency and thus proved to be suitable for the industrial production of eplerenone.

CONCLUSIONS

In this study, an *A. ochraceus* recombinant strain MF010 with high CYP monooxygenase expression was constructed through homologous recombination. The biocatalytic rate of this strain was 17–18 % higher than that of the original strain MF018 without the addition of any organic solvent or surfactant. Its biocatalytic time was also shorter by more than 30 h compared with that of the MF018 strain. The MF010 strain has potential application value in the industrial production of eplerenone as demonstrated by its high biocatalytic efficiency for the production of 11α-hydroxycanrenone.

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