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Heterologous Expression of *Rhizopus Oryzae CYP509C12* Gene in *Rhizopus Nigricans* Enhances Reactive Oxygen Species Production and 11α-Hydroxylation Rate of 16α, 17-Epoxyprogesterone

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ABSTRACT

The 11α-hydroxylation of 16α, 17-epoxyprogesterone (EP) catalyzed by *Rhizopus nigricans* is crucial for the steroid industry. However, lower conversion rate of the biohydroxylation restricts its potential industrial application. The 11α-steroid hydroxylase CYP509C12 from *R. oryzae* were reported to play a crucial role in the 11α-hydroxylation in recombinant fission yeast. In the present study, the *CYP509C12* of *R. oryzae* (*RoCYP*) was introduced into *R. nigricans* using the liposome-mediated mycelial transformation. Heterologous expression of *RoCYP* resulted in increased fungal growth and improved intracellular reactive oxygen species content in *R. nigricans*. The H_2O_2 levels in *RoCYP* transformants were approximately 2-folder that of the *R. nigricans* wild type (RnWT) strain, with the superoxide dismutase activities increased approximately 45% and catalase activities decreased approximately 68%. Furthermore, the 11α-hydroxylation rates of EP in *RoCYP* transformants (C4, C6 and C9) were 39.7%, 38.3% and 38.7%, which were 12.1%, 8.2% and 9.4% higher than the rate of the RnWT strain, respectively. This paper investigated the effect of heterologous expression of *RoCYP* in *R. nigricans*, providing an effective genetic method to construct the engineered strains for steroid industry.

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1. Introduction

Steroid compounds are terpenoids lipids containing the gonane nucleus of four cycloalkane rings [1]. In recent years, steroid hormones and their derivatives have been widely used in the pharmaceutical industry, mainly as immunosuppressive, anti-inflammatory, anti-rheumatic, progestational, diuretic, sedative, anabolic and contraceptive agents [2,3]. In addition, they are also used to treat hormone-dependent forms of breast and prostate cancer [1], osteoporosis, HIV infection and declared AIDS [4]. Currently, steroids constitute a vital part of the active ingredients in pharmaceuticals and intermediates used to produce medicines, and represent one of the largest sectors in the pharmaceutical industry [1].

Hydroxylation is one of the most important and commonly used reactions in the steroid industry, with the introduction of 11α-hydroxy to steroids by *Rhizopus* spp. being a great success in steroid biotransformation [1,5]. The 11α-hydroxylation performed by *R. nigricans* has been used in the pharmaceutical industry for more than a half century. However, the low conversion rate of 11α-hydroxylation conducted by *R. nigricans* is not sufficient for production scales [2].

The hydroxylation of the steroid skeleton structure is catalyzed by a family of enzymes, mainly the cytochrome P450s (CYPs) of the fungi [6]. The steroid hydroxylation systems in fungi are typical eukaryotic two-component systems that consist of a NADPH-CPR (cytochrome P450 reductase) and a cytochrome P450 monooxygenase [7,8]. The CYPs are external monooxygenases, in that they catalyze the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water [8]. The two main classes of CYPs are the mitochondrial/bacterial type and the microsomal type [7]. Microsomal CYPs are membrane bound and accept electrons from a microsomal CPR, which is an essential redox partner of CYPs, and whose function is to accept two electrons from NADPH and transfer them sequentially to CYPs [9,10]. The activities of CYP and CPR are crucial for various oxidation reactions performed by microsomal enzymes [11]. To date, many CYPs and CPRs have been cloned from fungal species, such as *R. oryzae* [12]. However, attempts to functionally characterize the purified or cloned steroid monooxygenases of *R. nigricans* have been unsuccessful so far.

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The 11α-hydroxylation of 16α, 17-epoxyprogesterone (EP) with *R. nigricans* (but not with *R. oryzae*) is an essential and important step in the process to produce 11α-hydroxy-16α, 17-epoxyprogesterone (HEP), and for decades, this has been a crucial step in the production of glucocorticoids in China [9]. Improving the conversion rate of 11α-hydroxylation is of great importance for the pharmaceutical industry. The 11α-steroid hydroxylase CYP509C12 from *R. oryzae* were reported to play a crucial role in the 11α-hydroxylation in recombinant fission yeast [12]. Considering the crucial role of CYPs in steroid hydroxylation systems, the function of *CYP509C12* of *R. oryzae* (*RoCYP*) was investigated in *R. nigricans* in the present study.

2. Materials and methods

2.1. Strains and culture conditions

Rhizopus nigricans LH21 from Henan Lihua Pharmaceutical (Anyang, Henan, China) was used as the wild type (RnWT) strain. *Rhizopus oryzae* CGMCC3.4826 was purchased from the China General Microbiological Culture Collection Center (CGMCC). The regeneration complete medium (RCM) for transformant screening contained (per liter, pH 7.0): glucose, 20 g; tryptone, 2 g; yeast extract, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.46 g; and K_2HPO_4, 1 g. The fermentation medium for *R. nigricans* contained (per liter, pH 4.5): glucose, 30 g; corn meal, 25 g; silkworm meal, 2 g; and $(NH_4)_2SO_4$, 1.6 g. *Escherichia coli* (DH5α) was used for plasmid amplification and was grown in lysogeny broth containing ampicillin (100 µg/ml) or kanamycin (50 µg/ml) as needed.

2.2. Cloning of RoCYP from Rhizopus oryzae

The sequence of *CYP509C12* (RO3G-05077.1) of *R. oryzae* were downloaded from the Broad Institute Database (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/MultiHome.html, 7.9.2009) and was used to design primers for amplification of *RoCYP*. The cDNA was synthesized from the total RNA isolated from *R. oryzae* treated with 300 µM EP as described previously [9] using the RT-PCR kit (Takara, Dalian, China). The coding sequence of putative *RoCYP* was amplified by PCR using the primers listed in Table 1, and cloned into the pMD19-T vector (Takara, Dalian, China) to generate pMD19T-CYP for sequencing.

2.3. Construction of fungal expression vectors

Plasmid pEGFP-C1 was used for the construction of expression vector (pC1-CYP) based on previously described study [13]. Briefly, the pMD19T-CYP vector was digested with *Age* I and *Kpn* I, and then *CYP* fragments were inserted into the pEGFP-C1 vector at the corresponding restriction sites, yielding the final expression vector pC1-CYP (Figure 1).

2.4. Transformation of Rhizopus nigricans

The pC1-CYP were introduced into *R. nigricans* using liposome-mediated mycelial transformation as described previously [13]. Briefly, 50 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 50 µl of 2 µg/ml pC1-CYP were mixed and placed on ice for 30 min. Then, 1 ml of the mycelial fragments suspension from *R. nigricans* mycelia ground with 0.6 M mannitol were added into the mixture of pC1-CYP and Lipofectamine 2000, mixed and placed on ice for another 30 min. Finally, the 100 µL of the transformation solution were plated on RCM medium supplemented with 300 µg/ml neomycin, and cultivated at 28 °C for 3 days. Colonies on the plate were randomly picked, and were used as the putative *RoCYP* transformants.

2.5. Transformants screening and subculture

The putative *RoCYP* transformants were subcultured on PDA plates containing 300 µg/ml neomycin at 28 °C three times, followed by purification through single spore isolation on PDA plates with 150 µg/ml neomycin. The isolates obtained were the *RoCYP* transformants and were used for further analysis.

2.6. Transformants identification

The integration of *RoCYP* gene in the *RoCYP* transformants was confirmed by PCR and RT-PCR using

Table 1. Oligonucleotide primers used.

Primer	Sequence (5′–3′)	Description
CYP-F	ATGATGGAAATGGCTGAATTTGC	Get the full length of *RoCYP* gene
CYP-R	TTAATAACGCTTTTTGAATATTAGTTCAA	
CYP-*Age*I-F	CGCTACCGGTCGCCACCATGATGGAAATGGCTGAATTTGC	Get *RoCYP* fragments for construction of pC1-CYP
CYP-*Kpn*I-R	GGGGTACCTTAATAACGCTTTTTGAATATTAGTTCAA	
sqRT-CYP-F	TTCATCGCTCAATGCCTATA	Detects the *RoCYP* expression
sqRT-CYP-R	GCCTTCATTAGCCAACGTA	
sqRT-Ssb1-F	TCTCCGTCGTCTCCGTTCTG	Detects the *Ssb1* expression
sqRT-Ssb1-R	GCCTTCATTAGCCAACGTA	

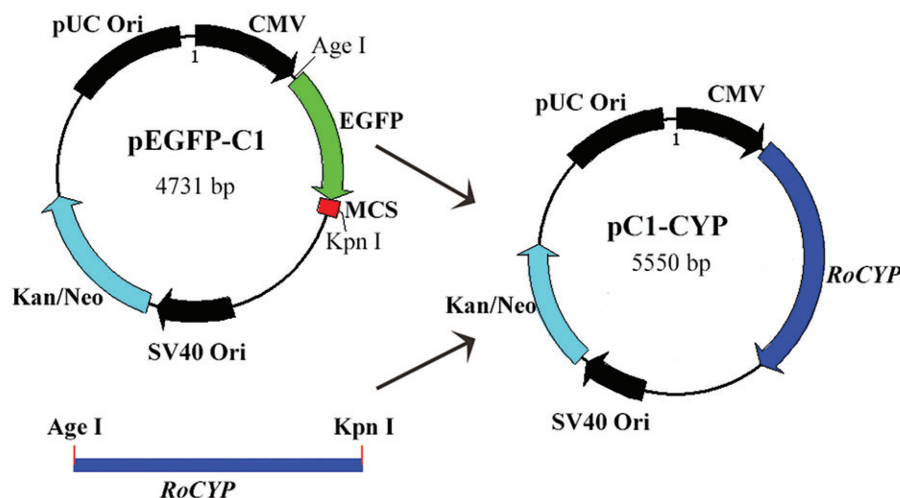

Figure 1. Construction of the *RoCYP* overexpression plasmid pC1-CYP. The *EGFP* gene of pEGFP-C1 is replaced by the *RoCYP* gene of *Rhizopus oryzae*. The *CYP* transcription is driven by the cytomegalovirus (CMV) promoter.

their genomic DNA and cDNA synthesized from their total RNA as templates, respectively. The expected PCR product size was 1,581 bp for *RoCYP* transformants. In addition, semi-quantitative RT-PCR was further carried out to screen transformants as described previously [13]. The housekeeping gene *Ssb1* (AY257252) was used as a control according to previous study [14].

2.7. ROS (reactive oxygen species) detection and enzymatic activity assays

The H_2O_2 content in the hyphae of *RoCYP* transformants from liquid PDA cultures was quantified by a commercial Hydrogen Peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). H_2O_2 bound to molybdenic acid forms a complex, measured at 405 nm, from which the content of H_2O_2 was calculated.

For the determination of enzymatic activity, mycelia of *RoCYP* transformants from liquid PDA cultures were ground in liquid N_2. Frozen mycelia (0.3 g) were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatant was immediately used for the following enzymatic assays. The protein content was determined according to the Bradford method, using BSA as a standard. The superoxide dismutase (SOD) activity and catalase (CAT) activity were measured as previously described [15]. To assay the SOD activity, the inhibition of the photochemical reduction of NBT was monitored at 560 nm. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of the reduction of NBT. The CAT activity was determined as the decomposition rate of H_2O_2 per minute at 240 nm as previously described [16].

2.8. Biotransformation reaction and HPLC measurements

The 1-ml spore solution (approximately 2×10^8 spores/ml) of the RnWT and *RoCYP* transformants was transferred into the fermentation medium (99 ml) in a 500-ml Erlenmeyer flask and cultured at 150 rpm for 22 h at 28 °C. Then, same amount of wet mycelia (1.0 g) were collected and transferred into new fermentation medium, followed by adding 2.0 g of EP and incubating for another 43 h. At the end of the biotransformation, the reaction products were extracted from the cells by circumfluence with acetone, and quantitatively evaluated by HPLC as described previously [17]. The HPLC measurements were performed in Xianju Pharmaceutical Company Ltd. (Taizhou, Zhejiang, China) as described elsewhere [12]. The biotransformation yield was used to evaluate the evaluation of HEP production efficiency and was calculated as the ratio between the peak areas (A) of the chromatograms of HEP and EP:

$$\text{Biotransformation yield} = \frac{\mathrm{A}\ (\mathrm{HEP})}{\mathrm{A(EP)} + \mathrm{A(HEP)}} \times 100$$

2.9. Statistical analysis

Data were expressed as the mean ± SE averaged from three independent sample measurements. Error bars represent standard deviations from the means of triplicates. The significance of samples was determined by analysis of variance, and differences between sample means were analyzed by one-way ANOVA and Tukey's honest significant difference test ($p < 0.05$).

3. Results

3.1. RT-PCR amplification of the RoCYP gene

With the reverse-transcribed cDNA of *R. oryzae* as the template, the coding sequences of *RoCYP* (MH125208) were amplified successfully. The results of sequence alignment showed that the amino acid sequence similarity was 96.96% between RoCYP cloned and CYP509C12 reported previously [12], with the conserved regions as the same (Figure S1 in Supplementary Material). This result indicates that the *RoCYP* obtained is highly consistent with the reported sequences and could be used for the next experiments.

3.2. Characterization of RoCYP transformants

To investigate the effect of the *RoCYP* gene on *R. nigricans*, the overexpression vector pC1-CYP was constructed (Figure 1). Liposome-mediated mycelial transformation was used to introduce pC1-CYP into *R. nigricans*. Approximately 20 transformants were screened from the neomycin-resistant RCM plates and subcultured three times. Then, six transformants were randomly selected and used for further analysis.

Multiplex PCR and RT-PCR analysis were carried out to confirm the presence of *RoCYP* gene in the *RoCYP* transformants, respectively. The results showed that there were obvious bands for the target genes from the genomic DNA and cDNA of *RoCYP* transformants (Figure 2), while there were no bands for the RnWT strain. These results indicate that the *RoCYP* gene were randomly integrated into the genome of *R. nigricans* and could be transcribed. Moreover, three *RoCYP* transformants (C4, C6 and C9) were further screened for the next experiments using semi-quantitative RT-PCR, as the relative expression of *RoCYP* in these selected transformants was higher (Figure 3).

3.3. The fungal growth was increased in RoCYP transformants

For an examination of the effect of *RoCYP* on the vegetative growth of *R. nigricans*, the mycelial diameters of the *RoCYP* transformants were determined. On PDA plates, the mycelial diameters of C4, C6 and C9 were increased by 17.8%, 16.1% and 16.7%, compared with that of the RnWT strain (Figure 4), respectively. Consistent with this result, the biomass of C4, C6 and C9 in PDB were improved by 71.1%, 59.3% and 74.1% compared with those of the RnWT strain (Figure 4). These results indicate that *RoCYP* increases the fungal growth of *R. nigricans*.

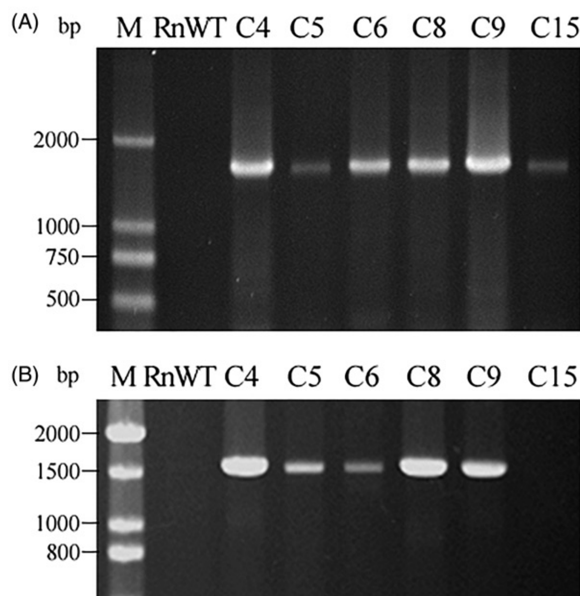

Figure 2. PCR and RT-PCR identification of putative *RoCYP* transformants. (A) PCR identification of *RoCYP* using genomic DNA of *RoCYP* transformants. (B) RT-PCR identification of *RoCYP* using cDNA of *RoCYP* transformants. M: DNA marker; RnWT: *Rhizopus nigricans* wild type; C4-C15: *RoCYP* transformants.

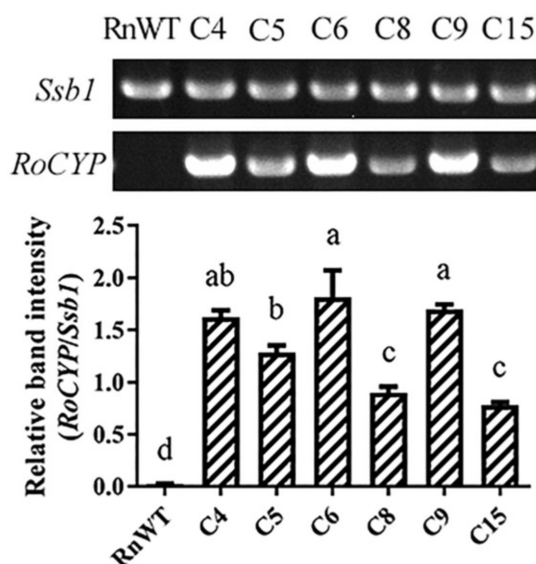

Figure 3. Expression of *RoCYP* gene determined by semi-quantitative RT-PCR. The amount of *RoCYP* mRNA, expressed as the ratio of densitometric measurement of the sample to the corresponding internal standard (*Ssb1*), is shown in their upper panels.

3.4. The intracellular ROS content was improved in RoCYP transformants

CYPs mediate ROS generation and are the main source of ROS [18]. To investigate whether *RoCYP* affect the intracellular ROS content of *R. nigricans*, the intracellular H_2O_2 content of the *RoCYP* transformants was tested using a spectrophotometer. As shown in Figure 5(A), the H_2O_2 levels in C4, C6 and C9 were 1.8-, 2.1- and 1.8-

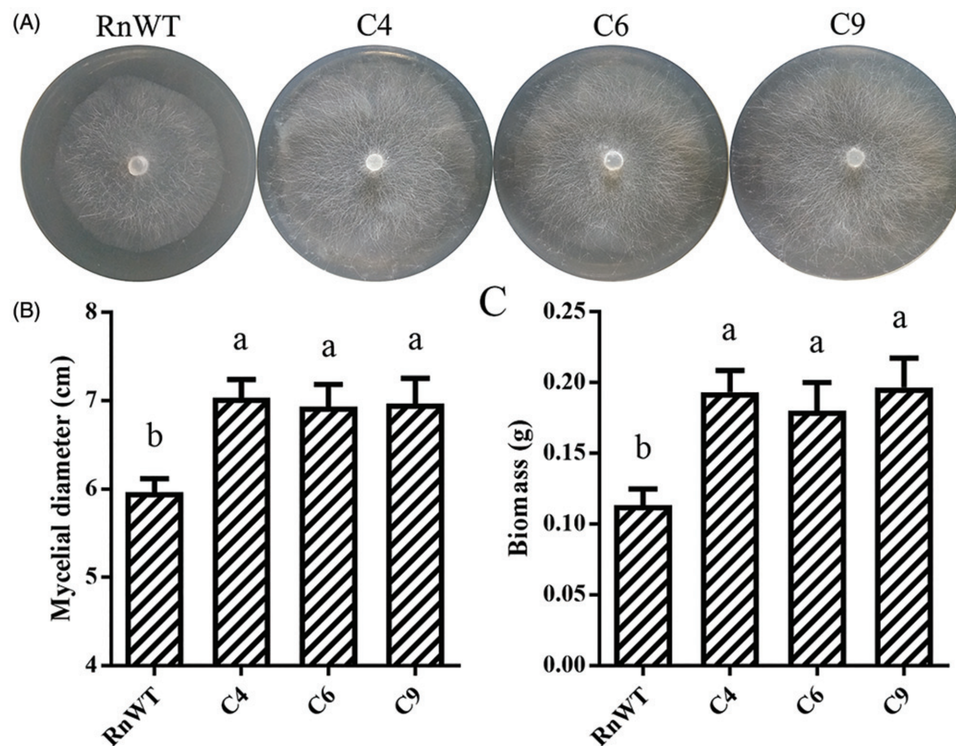

Figure 4. Fungal growth and biomass measurements of *RoCYP* transformants. (A) The tested strains were cultured on PDA at 28 °C for 24 h. (B) The mycelial diameter of the tested strains. (C) The biomass of the tested strains in PDB at 28 °C for 24 h.

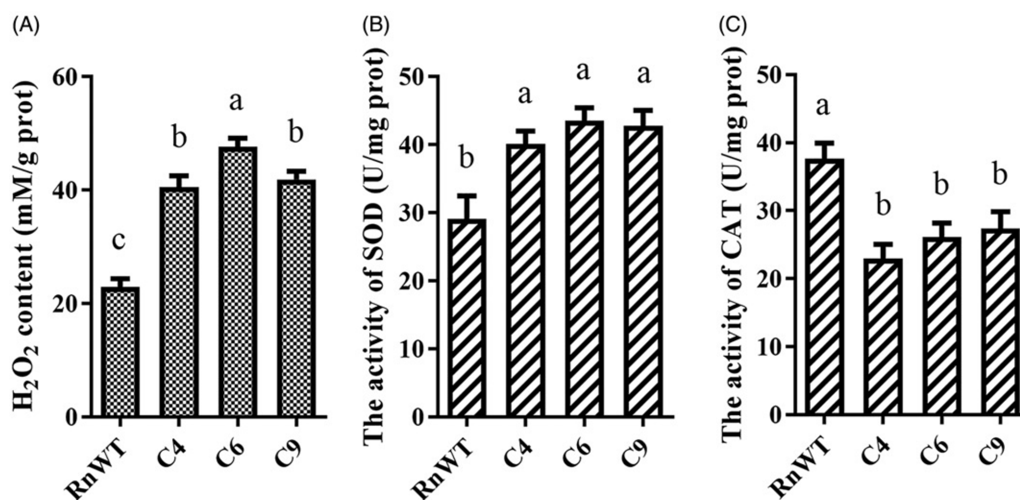

Figure 5. The intracellular ROS content and the activities of ROS-related enzymes in *RoCYP* transformants. (A) The intracellular H_2O_2 levels in the tested strains. (B) The SOD activity in the tested strains. (C) The CAT activity in the tested strains.

folder that of the RnWT strain, respectively. Moreover, the activity of two major antioxidant enzymes, SOD and CAT, was analyzed to further understand how *RoCYP* affects the ROS content. The SOD activities of C4, C6 and C9 were significantly increased by 38%, 50% and 47% compared with that of the RnWT strain, respectively (Figure 5(B)). However, the CAT activities of C4, C6 and C9 were 61%, 69% and 73% of the CAT activity of the RnWT strain (Figure 5(C)). These results imply that *RoCYP* increased the intracellular ROS content of *R. nigricans*.

3.5. The biotransformation abilities were higher in RoCYP *transformants*

The effect of *RoCYP* on the 11α-hydroxylation rate of *R. nigricans* was determined. With the addition of 2% EP in the fermentation medium, the biotransformation rates of C4 (39.7%), C6 (38.3%) and C9 (38.7%) were increased by 12.1%, 8.2% and 9.4%, compared with that of the RnWT strain, respectively (Figure 6). This result indicates that *RoCYP* has a promotion effect on the 11α-hydroxylation rate of *R. nigricans*.

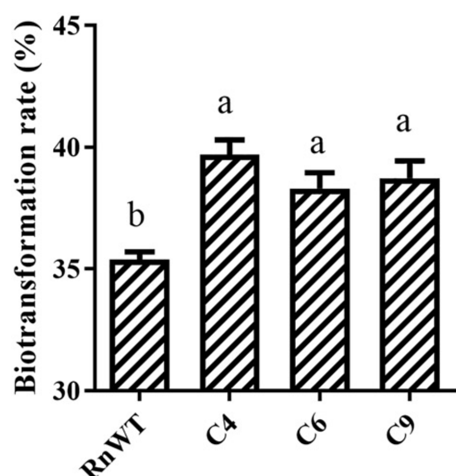

Figure 6. The biotransformation activities of *RoCYP* transformants.

4. Discussion

The lower conversion rate of the biohydroxylation is the bottleneck of 11α-hydroxylation of EP by *R. nigricans* which is used in steroid industry [9]. Cytochrome P450 monooxygenase was suggested to play a crucial role in steroid hydroxylation conducted by microorganisms [19,20]. Heterologous expression has become an efficient way to improve the conversion rate of steroid hydroxylation [1]. Here, the gene of 11α-steroid hydroxylase (*RoCYP*) was introduced into *R. nigricans*, and resulted in an enhancement of the 11α-hydroxylation rate of EP.

Researchers have explored and established various methods to improve the conversion rate of steroid hydroxylation, which could be sorted into two main categories: fermentation optimization and genetic modification. For fermentation optimization, conventional approaches to improve bioconversions were based on the micronization of substrate particles [21], surfactant-facilitated emulsification [22], the application of two-phase systems with organic solvents, liquid polymers, or cyclodextrins [5], as well as combinations of different methods [1]. For genetic modification, significant progress was made in the field of heterologous expression of steroidogenic P450 enzymes in microorganisms [11]. Co-expression of 11α-hydroxylase (CYP509C12) with its natural redox partner (RoCPR1) from *R. oryzae* in the fission yeast host showed a 7-fold enhancement in the 11α-hydroxylation rate compared with expression of CYP509C12 alone [12]. Soon after, the *RoCPR1* of *R. oryzae* was introduced into *R. nigrican*, and resulted in an improvement of the 11α-hydroxylation rate by 7.06% at the addition of 1% EP [9]. Here, we introduced the *RoCYP* into *R. nigricans*, and the 11α-hydroxylation rate of *RoCYP* transformants was significantly increased by 8.2 ~ 12.1% at the addition of 2% EP, implying that *RoCYP* also has a promotion effect on the 11α-hydroxylation rate of EP in *R. nigricans*. Co-expression of *RoCYP* and *RoCPR1* in *R. nigricans* might further improve the 11α-hydroxylation rate, which should be explored in the future.

Numerous studies have suggested that CYP metabolism plays a crucial role in intracellular ROS generation. In the endoplasmic reticulum, the major source of ROS is the microsomal monooxygenase (MMO) system, whose main function is mixed-function oxygenation of xenobiotics and some endogenous substrates [23]. The MMO system also leads to the release of large amounts of ROS from the P450 enzyme, such as superoxide anion radical and H_2O_2 [24]. CYP2E1 is believed to contribute to ROS production in different pathophysiological situations among various CYPs [25,26]. Consistently, heterologous expression of *RoCYP* in *R. nigricans* resulted in an improvement of ROS production. CYP-mediated estrogen metabolites was reported to produce high ROS concentrations [27]. Similarly, the improved biotransformation abilities in *RoCYP* transformants accompanied the increased ROS accumulation in the present study, and there might be an association between the increased biotransformation rates and improved intracellular ROS content in *RoCYP* transformants. Nevertheless, the mechanisms of *RoCYP* regulating intracellular ROS species need to be examined in depth in the future.

In summary, the *RoCYP* was introduced into *R. nigricans* using liposome-mediated mycelial transformation, and the 11α-hydroxylation rates of the *RoCYP* transformants were significantly improved. To our knowledge, this is the first report of the overexpression of *RoCYP* in *R. nigricans*, providing an effective genetic method to improve the productivity of HEP.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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