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# Improving bioconversion of eugenol to coniferyl alcohol by *in situ* eliminating harmful $H_2O_2$

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ABSTRACT

Coniferyl alcohol is a valuable chemical. However, the current approaches to obtain coniferyl alcohol are either unefficient or expensive. *Penicillium simplicissimum* vanillyl alcohol oxidase (PsVAO) can be used to produce coniferyl alcohol. However, PsVAO intrinsically produces harmful byproduct $H_2O_2$. Utilizing catalase to decompose $H_2O_2$ is a potential straightforward approach; however, catalase can also exhibit peroxidase activity to facilitate coniferyl alcohol over-oxidation. In this study, catalases exhibiting both high catalase activity and low peroxidase activity were found out, and introduced into the bioconversion systems. Our results showed that eliminating $H_2O_2$ *in situ* released $H_2O_2$ inhibition of PsVAO, improved coniferyl alcohol production and eliminated coniferyl alcohol over-oxidation. Finally, coniferyl alcohol titer, molar yield, and productivity reached 22.9 g/L, 78.7%, and 0.5 g/(L × h) respectively. An efficient coniferyl alcohol production method was developed by overcoming the intrinsic disadvantages of PsVAO.

## 1. Introduction

Coniferyl alcohol is a compound with high market value. As a natural monolignol, coniferyl alcohol is widely used to elucidate the biosynthesis processes and chemical structures of a variety of plant metabolites, including lignins (Watts et al., 2011), lignans (Ono et al., 2006), and flavonolignans (Althagafy et al., 2013). In addition to basic phytochemical studies, coniferyl alcohol is also used to synthesize various valuable chemicals, such as silybin (Althagafy et al., 2013), pinoresinol, sesamin (Ono et al., 2006), ferulic acid, and vanillin (Overhage et al., 2003). Because these chemicals generally are found in low concentrations in their originating plants, as well as harboring complicated chemical structures and synthetic processes, both their botanical extraction and chemical synthesis present potential challenges (Chen et al., 2017). Reduction of ethyl ferulate is the most commonly used synthetic route to coniferyl alcohol, however, this method suffers from expensive starting materials and catalyst, harsh reaction condition, and low overall yield (Kirk and Brunow, 1988; Quideau and Ralph, 1992). For instance, in the improved method, coniferyl alcohol was synthesized by reduction of ethyl ferulate using diisobutylaluminum hydride (DIBAL-H) in toluene as reducing agent (Quideau and Ralph, 1992). Generating these chemicals by bioconversion shows many advantages over the methods described above (de Carvalho, 2017).

Reconstruction of the original synthetic pathway in microorganisms is a promising approach for producing valuable natural plant products (Chemler and Koffas, 2008). However, the original coniferyl alcohol synthesis pathway involves eight enzymes, including two cytochrome *c* P450 enzymes, which are difficult to actively express in prokaryotic microorganisms (Caswell et al., 2013). Although redesign of the pathway eliminated the necessity for cytochrome *c* P450, the pathway reconstruction remains time consuming and complicated (Wang et al., 2015). Moreover, the shikimate pathway, which provides the substrate for coniferyl alcohol synthesis, is accurately and strictly regulated (Bongaerts et al., 2001). According to previous studies, 124.9 mg/L coniferyl alcohol can be obtained by reconstructing the *de novo* pathway in microorganisms (Chen et al., 2017). However, this remains far from adequate for commercial applications. Therefore, producing coniferyl alcohol from inexpensive precursors represents a promising alternative to the *de novo* biosynthesis of this important natural plant product (de Carvalho, 2017).

Eugenol is a natural and inexpensive (~5 USD/kg) substrate, and

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frequently used in the food and cosmetic industries (Overhage et al., 2003; Overhage et al., 2006). *Penicillium simplicissimum* vanillyl alcohol oxidase (PsVAO) can oxidize a wide range of *para*-substituted phenols, including eugenol (Fraaije et al., 1995). PsVAO oxidizes eugenol to coniferyl alcohol, producing equimolar $H_2O_2$ as a byproduct. Although endogenous *Escherichia coli* catalases and peroxidases can scavenge $H_2O_2$, rapid accumulation of $H_2O_2$ during conversion exceeds the threshold of this ability (Korshunov and Imlay, 2010; Ricklefs et al., 2015). Excess $H_2O_2$ results in feedback inhibition of PsVAO and is toxic to the host cell (Lv et al., 2017). Moreover, $H_2O_2$ serves as an electron acceptor in coniferyl alcohol polymerization catalyzed by peroxidase, and endogenous *E. coli* peroxidase is capable of consuming coniferyl alcohol in the presence of excess $H_2O_2$ (Korshunov and Imlay, 2010). Therefore, eliminating excess $H_2O_2$ from the reaction system can potentially relieve feedback inhibition of PsVAO and prevent consumption of coniferyl alcohol.

PsVAO is a cofactor (flavin adenine dinucleotide)-dependent enzyme. Therefore, whole-cell catalysis is needed to support cofactor supply and regeneration processes (Kara et al., 2014). The enhancement of catalase to the reaction system can improve the decomposition of $H_2O_2$ to $H_2O$ and $O_2$, thereby protecting PsVAO from inhibition and host cells from damage. In this study, catalases from different microorganisms were screened and the enzymes with high catalase activity and low peroxidase activity were introduced into the bioconversion systems. Our results showed that this system enhanced bioconversion of eugenol to coniferyl alcohol by protecting PsVAO from inhibition and preventing coniferyl alcohol from over-oxidation. After condition optimization and scale up, coniferyl alcohol titer, molar yield, and productivity reached 22.9 g/L, 78.7%, and 0.5 g/(L × h), respectively. Our findings and the methods described provide the most promising current biological route for producing coniferyl alcohol on an industrial scale.

## 2. Materials and methods

### 2.1. Genes, plasmids, and strains

The ePathBrick vector pET28a(PB) was used for protein expression. *E. coli* JM109 was used for plasmid construction, maintenance, and propagation. *E. coli* BL21 (DE3) was used for protein expression. Catalase genes were amplified from the genomic DNA of different microorganisms. The backbone of pET28a(PB) was amplified with primer pair pET28a(PB) $F_{CE}$/pET28a(PB) $R_{CE}$. Primers are listed in Table 1. Catalase genes were subcloned into pET28a(PB) between restriction sites *Nco*I/*Eco*RI using the ClonExpress II One Step cloning kit (Vazyme Biotech Co., Ltd, Nanjing, China) according to manufacturer protocol. All the sequences were verified by Sanger sequencing (Sangon Biotech, Shanghai, China). *PsVAO* and catalase genes were assembled into their monocistronic form using the ePathBrick method (Xu et al., 2012). Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells for protein expression (Table 2).

**Table 1**
Primers used in this study.

| Primers | Sequence (5′-3′) |
|---|---|
| Bs_CAT F | CTTTAAGAAGGAGATATACCatgagtgatgaccaaaacaa |
| Bs_CAT R | CTTGTAGACGGAGCTCGAATtcaaattcgtctatcccaat |
| Ec_CAT F | CTTTAAGAAGGAGATATACCatgtcgcaacataacgaaaa |
| Ec_CAT R | CTTGTAGACGGAGCTCGAATtcaggcaggaattttgtcaa |
| Pp_CAT F | CTTTAAGAAGGAGATATACCatgcccagcaagaaaacgga |
| Pp_CAT R | CTTGTAGACGGAGCTCGAATtcaggcaggaacagcttcag |
| Sa_CAT F | CTTTAAGAAGGAGATATACCatgcaagatatttacttttt |
| Sa_CAT R | CTTGTAGACGGAGCTCGAATttatttttcaaagttttcgt |
| Sc_CTA1 F | CTTTAAGAAGGAGATATACCatgtcgaaattgggacaaga |
| Sc_CTA1 R | CTTGTAGACGGAGCTCGAATtcaaaatttggagttactcg |
| Sc_CTT1 F | CTTTAAGAAGGAGATATACCatgaacgtgttcggtaaaaa |
| Sc_CTT1 R | CTTGTAGACGGAGCTCGAATttaattggcacttgcaatgg |
| pET28a(PB) $F_{CE}$ | GGTATATCTCCTTCTTAAAGTTAAAC |
| pET28a(PB) $R_{CE}$ | ATTCGAGCTCCGTCTACAAGCTTGC |

Capital letters represent sequences overlapping those of the pET28a(PB) sequence.

### 2.2. $H_2O_2$-inhibition assay

Recombinant strPsVAO was washed and resuspended in phosphate-buffered saline (PBS, 50 mM $NaH_2PO_4$-$Na_2HPO_4$ (pH7.0)) and homogenized by ultrasonication on ice to release recombinant PsVAO·$H_2O_2$ was added to the cell homogenate to different final concentrations as indicated in Fig. 1. An equal volume of PBS was used as a control. All assays were performed in 1-mL volumes and in 50-mL centrifuge tubes. The mixtures were incubated at 37 °C and 220 rpm for 30 min before addition of a final concentration of 0.5% (v/v, or 5.3 g/L) eugenol. Coniferyl alcohol and pinoresinol were analyzed using a previously described method with moderate modifications (Lv et al., 2017). PsVAO activity was determined by calculating coniferyl alcohol productivity over 30 min.

### 2.3. Gene expression

Catalase genes from different microorganisms were subcloned into the ePathBrich vector pET28a(PB) (Lv et al., 2017). Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells for protein expression, which was performed in 25 mL Terrific broth (TB) medium in 250-mL shaking flasks. After growth to log phase, the recombinant strains were pre-cooled to 25 °C. A final concentration of 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression. The recombinant strains were cultured at 25 °C for another 6 h for protein expression.

Cells were lysed and recombinant protein released by ultrasonication on ice. The debris was removed by centrifugation, and the supernatant was used to determine protein concentration, expression, and activity. Protein concentration was determined using an enhanced BCA protein assay kit (Beyotime, Nantong, China). Recombinant protein expression was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.4. Enzyme assay

Catalase activity was determined with a catalase assay kit (Beyotime) (Wang et al., 2013a). One unit of catalase activity was defined as 1 μM $H_2O_2$ degradation in 1 min at 45 °C and pH 9.0. Peroxidase activity was analyzed by 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay with moderate modifications (Naeaetsaari et al., 2014). Reaction kinetics was determined by reading the absorbance at 405 nm ($A_{405}$). *E. coli* BL21 (DE3) cells harboring blank pET28a(PB) were used as the blank control. All catalase and peroxidase assays were performed independently in triplicate.

### 2.5. Bioconversion condition optimization and scale up

To determine the effect of pH and temperature on PsVAO activity, bioconversion was performed in 5-mL volumes in 50-mL centrifuge tubes. Recombinant protein expression was performed as described in Section 2.4, and cells were harvested by centrifugation at 4 °C and 8000*g* for 15 min. The cells were resuspended in buffers with different pH values, and 50 mM PBS (pH 4.5, 5.5, 6.0, 7.0, 8.0, or 9.0) or 50 mM glycine-NaOH (pH 10.0 or 11.0) was used to buffer the reaction. A final amount of $OD_{600}$ = 10.0 ± 0.5 cells was used as the catalyst. A final concentration of 0.5% (v/v, 5.3 g/L) eugenol was used as the substrate. The reaction was performed at 25 °C, 30 °C, 37 °C, 45 °C, 50 °C, or 55 °C at 220 rpm for 2 h. The reaction medium was diluted 200-fold with methanol and filtered using a 0.22-μm membrane (Sangon Biotech, Shanghai, China). Coniferyl alcohol and pinoresinol were analyzed by