Efficient Biosynthesis of 10-Hydroxy-2-decenoic Acid Using a NAD(P)H Regeneration P450 System and Whole-Cell Catalytic Biosynthesis

Li Wang, Leilei Wang, Ruiming Wang, Zhaoyun Wang, Junqing Wang, Haibo Yuan, Jing Su, Yan Li, Suzhen Yang, and Tingting Han

ABSTRACT: 10-Hydroxy-2-decenoic acid (10-HDA) is an \(\alpha,\beta\)-unsaturated medium-chain carboxylic acid containing a terminal hydroxyl group. It has various unique properties and great economic value. We improved the two-step biosynthesis method of 10-HDA. The conversion rate of the intermediate product trans-2-decenoic acid in the first step of 10-HDA synthesis could reach 93.1 ± 1.3% by combining transporter overexpression and permeation technology strategies. Moreover, the extracellular trans-2-decenoic acid content was five times greater than the intracellular content when 2.0% (v/v) triton X-100 and 1.2% (v/v) tween-80 were each used. In the second step of 10-HDA synthesis, we regenerated NAD(P)H by overexpressing a glucose dehydrogenase with the P450 enzyme (CYP153A33/M228L-CPRBM3) in Escherichia coli, improving the catalytic performance of the trans-2-decenoic acid terminal hydroxylation. Finally, the yield of 10-HDA was 486.5 mg/L using decanoic acid as the substrate with two-step continuous biosynthesis. Our research provides a simplified production strategy to promote the two-step continuous whole-cell catalytic biosynthesis of 10-HDA and other \(\alpha,\beta\)-unsaturated carboxylic acid derivatives.

INTRODUCTION

10-Hydroxy-2-decenoic acid (10-HDA) is a terminal hydroxylated medium-chain \(\alpha,\beta\)-unsaturated carboxylic acid. This chemical performs many unique physiological activities, such as antibacterial, antioxidative, anti-inflammatory, immune regulation, and antitumor effects. Therefore, 10-HDA has high economic value and broad application prospects. In nature, the 10-HDA has only been found in royal jelly, and it is the most important fatty acid in royal jelly. The traditional physical extraction method is associated with high production costs, thus resulting in failure to meet the market demand. Since the 1960s, various methods for the chemical synthesis of 10-HDA have appeared consecutively, which include Wittig reagent synthesis, ozonation, bromination elimination, Knoevenagel condensation, and growing carbon chain synthesis. However, chemical synthesis methods have disadvantages, such as complicated operation, prone to environmental pollution, and low controllability of the reaction process. Compared with physical extraction and chemical synthesis, biocatalysis is highly selective and environmentally friendly and has thus aroused great interest. Recently, the biosynthesis of 10-HDA was reported using decanoic acid as a substrate via two-step whole-cell catalysis. In the first step, decanoic acid was converted to trans-2-decenoic acid through the modified \(\beta\)-oxidation pathway in Escherichia coli. In the second step, CYP153A33/M228L-CPRBM3 efficiently catalyzed the conversion of trans-2-decenoic acid to 10-HDA. In this process, trans-2-decenoic acid is a crucial intermediate product, and it reached a yield of 312 mg/L, and the conversion rate was 62.4% in the previous study. To the best of our knowledge, few...
reports on the biosynthesis of trans-2-decenoic acid are available. Kim et al. reported that trace amounts of 2-decenoic acid can be detected in the byproduct of the biosynthetic production of crotonic acid, and the yield was only 9.5 mg/L.10 In engineered strains, fatty acid often causes the physiological activity of the host to be disturbed and results in a reduced yield of fatty acid.11 A promising strategy is to accelerate the fatty acid transport across the cell membrane. Therefore, it is an effective and economical strategy to regenerate NAD(P)H by overexpressing the cofactor regenerating enzyme, thus increasing the availability of cofactors and improving the catalytic performance of P450.22

In this study, we first combined the transporter overexpression and permeation technology strategy to improve the conversion rate of trans-2-decenoic acid using genetically engineered strains and next explored the difference in the intracellular and extracellular production of the intermediate product. In the second step of 10-HDA synthesis, we regenerated the cofactor NAD(P)H by coupling the expression of P450 enzymes (CYP153A33/M228L-CPR<sub>BM3</sub>) with glucose dehydrogenase (GDH) derived from Bacillus subtilis 168 (B. subtilis 168), which increased the reaction efficiency of the conversion of trans-2-decenoic acid to 10-HDA. During the synthesis method, the reaction solution of the first step was directly applied to the second step catalytic system without crushing the cells, which simplified the fermentation steps and reduced the 10-HDA production time.

### Table 1. List of Primers

| primer names          | primer base sequence (5′ → 3′)                                      |
|-----------------------|---------------------------------------------------------------------|
| Pf_fadL(Xhol)         | gtxgacgtggtctacgtggtcGAAGTAGCACCAAATCCTGGTTTACA                   |
| Pr_fadL(PacI)         | tgccgacgtggtctacgtggtcTAACAGGACGCCGTTAAATTTGATAGACC                |
| Pr_sumo-<i>5</i>ylid(BamH1) | tcatcacacagcgtggtcATGTCGGACTCAGAAGTCAATCAA                        |
| Pr_sumo-<i>5</i>ylid(HindIII) | gcattagcgcagcagcgtggtcCTCGAAGTACACCGGCGG                      |
| Pf_mdtE(NotI)         | taagagagagatacatagtATGACAAGAAAGAAAGCTGTATATCACC                 |
| Pr_mdtE(Xhol)         | gttcttacagctcggtTTATGTGTTCGATCTGGTCTG                           |
| Pf_acrE(NotI)         | taagagagagatacatagtATGACAAGAAAGAAAGCTGTATATCACC                 |
| Pr_acrE(BamHI)        | gcctagcgcagcgtggtcCTCGAAGTACACCGGCGG                      |
| Pr_sumo-<i>5</i>ylid(AseI) | tcgaatctcgacgctcgcccAGTTCGGAACCTGACATCAAC                        |
| Pr_sumo-<i>5</i>ylid(HindIII) | gcattagcgcagcagcgtggtcCTCGAAGTACACCGGCGG                      |
| Pf_CYP153(Ndel)       | gttcccgtctacgtcggTATTGTGTTCGATCTGGTCTG                           |
| Pf_CYP153              | gcctagcgcagcgtggtcCTCGAAGTACACCGGCGG                      |
| Pr_GDH                | aattttactccatCCCAGCCCACACGGCAG                                    |
| Pr_GDH(Xhol)          | gttcttacagctcggtTTATGTGTTCGATCTGGTCTG                           |

### Table 2. List of Engineering Strain and Plasmid Names

| strain/plasmid | description | source (ref) |
|----------------|-------------|-------------|
| E. coli BL21(DE3) (ΔfadBJR) | ∆fadB::FRT ΔfadD::FRT ΔfadK::FRT sequential deletion of fadB, fadD, and fadK harboring a ΔDE3 lysogen | 9 |
| pCDFDuet-1::<i>5</i>fadE<sup>36</sup>MACS | pCDFDuet-1 Carry <i>5</i>fadE and <sup>36</sup>MACS gene | 9 |
| pET-28a-sumo-<i>5</i>ylid | pET-28a Carry <i>5</i>ylid gene | 9 |
| pET-21b-CPY153A33/M228L-CPR<sub>BM3</sub> | pET-21b Carry CYP153A33/M228L-CPR<sub>BM3</sub> gene | 9 |
| pETDuet-1::<i>5</i>ylid<sub>fadL</sub> | pETDuet-1 Carry <i>5</i>ylid and fadL gene | this study |
| pETDuet-1::<i>5</i>ylid<sub>acrE</sub> | pETDuet-1 Carry acrE and <i>5</i>ylid and mdtE gene | this study |
| pETDuet-1::<i>5</i>ylid<sub>mdtE</sub> | pETDuet-1 Carry mdtE and <i>5</i>ylid gene | this study |
| pETDuet-1::<i>5</i>ylid<sub>GDH</sub> | pETDuet-1 Carry GDH and <i>5</i>ylid gene | this study |
| E. coli (ΔfadBJR/fadD-MACS/ydil) | E. coli BL21(DE3) (ΔfadBJR)/pCDFDuet-1::<i>5</i>fadE<sup>36</sup>MACS/pET-28a-sumo-<i>5</i>ylid | 9 |
| E. coli (CYP153A33/M228L-CPR<sub>BM3</sub>) | E. coli BL21(DE3) (pET-21b-CPY153A33/M228L-CPR<sub>BM3</sub>) | 9 |
| E. coli (CYP153A33/M228L-CPR<sub>BM3</sub>/GDH) | E. coli BL21(DE3) (pET-28a-CPY153A33/M228L-CPR<sub>BM3</sub>/GDH) | this study |
MATERIALS AND METHODS

Materials, Bacterial Strains, and Plasmids. The chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA ligase and single-fragment seamless cloning kits used in this study were all purchased from Nazyme (Nanjing, China). The plasmids pCDFDuet-1,\(^\text{pCDFDuet-1}\), pET-28a-sumo-\(^\text{pET-28a-sumo}\), pET-28a-\(^\text{pET-28a}\), pETDuet-1-\(^\text{pETDuet-1}\), CYP153A33/M228L-CPRBM3-GDH, pETDuet-1-\(^\text{pETDuet-1}\), and pET-28a-CYP153A33/M228L-CPRBM3-GDH were all constructed as mentioned previously.\(^\text{9}\) The first two plasmids were used for the synthesis of trans-2-decenoic acid. The third plasmid was used for 10-HDA from trans-2-decenoic acid. Based on these plasmids, pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\), pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\), pET-28a-CYP153A33/M228L-CPRBM3-GDH, and pET-28a-CYP153A33/M228L-CPRBM3-GDH were successfully constructed. The plasmids pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\), pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\), pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\), and pETDuet-1-sumo-\(^\text{pETDuet-1-sumo}\) exhibited the coexpression of thioesterase (CYP153A33/M228L)-\(^\text{CYP153A33/M228L}\) with different fatty acid transporters. The plasmid pET-28a-CYP153A33/M228L-CPRBM3-GDH contained coexpression of the P450 enzyme (CYP153A33/M228L-CPRBM3) encoding gene and the GDH gene from \(B. \) subtilis 168. The latter gene is responsible for the regeneration of NAD(P)H. The primers used in this study to construct these plasmids are listed in Table 1. \(E. \) coli BL21(DE3) (\(\Delta fadBJR\)) was used as a starting strain for the synthesis of trans-2-decenoic acid from decanoic acid, which comprised the deletion of three genes (\(fadB, fadJ, \) and \(fadr\)) involved in \(\beta\)-oxidation.\(^\text{9}\) All engineered strains in this study contained different plasmids (Table 2), which were all confirmed using DNA sequencing (Sangon Biotech).

Culture Medium and Conditions. The engineered strains were fermented in Luria–Bertani (LB) medium and incubated at 37 °C for 200 rpm. The corresponding antibiotics, streptomycin (40 \(\mu\)g/mL), kanamycin (50 \(\mu\)g/mL), and ampicillin (100 \(\mu\)g/mL), were added to the LB medium to culture engineered strains with different plasmids. After the optical density at 600 nm (\(OD_{600}\)) reached 0.8–1.2, the culture temperature was changed to 20 °C. Then, isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG; 0.5 mM) was added to the culture medium, and the engineered strains were subjected to continuous incubation for 20 h. In \(E. \) coli (CYP153A33/M228L-CPRBM3) and \(E. \) coli (CYP153A33/M228L-CPRBM3-GDH), 0.5 mM S-aminolevulinic acid hydrochloride and 0.5 mM FeCl\(_\text{2}\) were added with IPTG, and cells were incubated at 20 °C for 20 h. The \(OD_{600}\) value was measured with an ultraviolet spectrophotometer; the cells were collected by centrifugation at 5500 rpm for 16 min. The cells were washed twice with deionized water to remove the remaining reagents in the fermentation broth. Finally, the collected cells were resuspended in a potassium phosphate buffer (0.1 M, pH 7.4) for the next cell reaction, and the wet cell mass of bacteria was 0.75 g/10 mL.

Permeabilization Treatment and Whole-Cell Catalysis System for Decanoic Acid to trans-2-Decenoic Acid. The cells were treated with a ranging concentration of permeabilization reagents, which included cetrizonium bromide (CTAB, 0.1–0.5 g/L), ethylenediaminetetraacetic acid (EDTA, 0.1–0.5 g/L), and colistin sulfate (0.2–1.4 g/L). Thereafter, the cells were gently stirred with a rotary shaker (200 rpm) at 30 °C for 20 min and washed twice with deionized water. The other permeabilization reagents, dimethyl sulfoxide (DMSO, 0.4–1.2%), triton X-100 (0.5–2.0%), and tween-80 (0.4–1.2%), were directly added to the reaction liquid system. The whole-cell catalysis system in this step comprised 30 mL of resuspended cells supplemented with 0.5 g/L decanoic acid, 1% glycerol, 0.4% glucose, 40 \(\mu\)g/mL streptomycin, and 50 \(\mu\)g/mL kanamycin. The reaction system was incubated at 30 °C with mild shaking at 200 rpm. All of aforementioned reactions were repeated more than three times.

Optimization of CYP153A33/M228L-CPRBM3-GDH Expression and Whole-Cell Catalysis System of trans-2-Decenoic Acid to 10-HDA. The induction conditions of \(E. \) coli (CYP153A33/M228L-CPRBM3-GDH) are important and have an important effect on the expression of heterologous proteins.\(^\text{2,21,22}\) The effect of the starting induction time was evaluated when the cell concentration reached an \(OD_{600}\) of 0.4, 0.6, 0.8, 1.0, and 1.2. The effect of IPTG concentrations was evaluated at 0.4, 0.5, 0.6, 0.7, and 0.8 mM. Finally, the effect of the induction time (12, 16, 20, and 24 h) on the coupling expression of CYP153A33/M228L-CPRBM3-GDH was analyzed. The whole-cell catalysis system of \(E. \) coli (CYP153A33/M228L-CPRBM3-GDH) was 30 mL, which included the resuspended cell pellet, trans-2-decenoic acid (final concentration, 0.5 g/L), glycerin (final concentration, 1%), glucose (final concentration, 0.4%), and 50 \(\mu\)g/mL kanamycin. The reaction system was sampled after 2, 4, 6, 8, 20, and 24 h, and the experiment was repeated three times.

Reaction Conditions for Whole-Cell Catalysis. Li et al. reported that whole cells can catalyze the production of 10-HDA from decanoic acid using two-step whole-cell catalysis.\(^\text{9}\) The whole-cell catalysis of decanoic acid to produce 10-HDA was performed as follows: The cultured recombinant bacteria \(E. \) coli (\(\Delta fadBJR/fadE-MACS/\)ydil) were permeated to prepare a whole-cell catalytic system, which was used to catalyze the conversion of decanoic acid to trans-2-decenoic acid. After 9 h of the reaction, the supernatant was collected by centrifugation and used in the following step. The engineered bacteria \(E. \) coli (CYP153A33/M228L-CPRBM3-GDH) were then collected, resuspended, and added to the supernatant resuspension solution collected in the previous step. The reaction system also contained 1% glycerol, 0.4% glucose, 0.1 \(\mu\)M FeSO\(_4\), and kanamycin (50 \(\mu\)g/mL). They were allowed to react at 200 rpm at 30 °C for 20 h. These reactions were repeated three times.

Fatty Acid Extraction and Analysis. The reaction mixture was centrifuged at 12,000 rpm for 10 min, the obtained supernatant was used to determine the extracellular fatty acid content, and the precipitate was used to determine the intracellular fatty acid content. The intracellular and extracellular production of fatty acids was analyzed using the fatty acid extraction and derivatization detection method of Li et al. via gas chromatography-mass spectrometry (GC-MS, Agilent).\(^\text{9}\)

RESULTS AND DISCUSSION

Role of Transporters in the Production of trans-2-Decenoic Acid. To identify the endogenous transporter that can accelerate trans-2-decenoic acid production, we constructed engineered strains of \(E. \) coli BL21(DE3) (\(\Delta fadBJR\)), consisting of two different plasmids. The first plasmid, pCDFDuet-1-\(^\text{pCDFDuet-1}\), was derived from our previous research,\(^\text{9}\) and the second plasmid was used to coexpress the thioesterase gene \(^\text{CYP153A33/M228L-CPRBM3-GDH}\) and the transporter gene (Figure S1). Here, we investigated two types of transporters. The first was the combination AcrE and MdtE transporters, which were...
derived from the resistance-nodulation cell division (RND) superfamily. They were used for improving the yield of medium-chain fatty acids (C6−C10). The second transporter was FadL, which is a fatty acid transporter of the E. coli cell outer membrane that can improve the biotransformation of various fatty acids. Finally, we constructed endogenous transporter recombinant plasmids, namely pETDuet-1-sumo-CtydiI-fadL and pETDuet-1-acrE-sumo-CtydiI-mdtE. We found that with the coexpression of acrE and mdtE in coordination, the total production of trans-2-decenoic acid could reach 370.8 mg/L, and the conversion rate reached 74.2% (Figure 1A). This is an improvement compared with those in the control group, for which the total production of trans-2-decenoic acid was 309.3 mg/L and the conversion rate was 61.9%. However, it did not result in a rise in the detection of trans-2-decenoic acid outside the cells (Figure 1B,C). We inferred that coexpression of the acrE and mdtE combination promoted the transport of the substrate decanoic acid into the cells, like that reported in a previous study, thereby favoring the production of trans-2-decenoic acid catalyzed by the intracellular enzymes, but the combined expression of the two transporters had low specificity for

Figure 1. Effect of transporters on the production of trans-2-decenoic acid. (A) Effect of resistant nodulation cell division family transporter (RND) and outer membrane protein on the production of total trans-2-decenoic acid. (B) Effect of RND family and outer membrane protein on the production of intracellular trans-2-decenoic acid. (C) Effect of RND family and outer membrane protein on the production of extracellular trans-2-decenoic acid. The control strain was E. coli (ΔfadBJR/fadE-MACS/ydiI) to study the effect of transporter engineering on medium-chain fatty acid production.

Figure 2. Effect of different permeabilization reagents on the engineered strain producing trans-2-decenoic acid. (A) Triton X-100. (B) Tween-80. (C) CTAB. (D) EDTA. (E) DMSO. (F) Colistin sulfate.
2-decenoic acid transport. Additionally, expression of the cell outer membrane protein FadL reduced the total concentration of \( \text{trans-2-decenoic acid} \) by 22.7\% (Figure 1A). This result showed that FadL had poor transport specificity for medium-chain fatty acids, and it was agreed to previous work that inferred FadL was mainly responsible for the transport of long-chain fatty acids (\( \text{C}_{16}-\text{C}_{18} \)).

**Comparison of Different Permeabilization Treatments Applied to \text{trans-2-Decenoic Acid Using Genetically Engineered Strains.}** Permeabilization treatment can induce changes in the permeability of bacterial cell membranes, which allows small molecules to enter cells freely. Park et al. reported that by screening and optimizing different combinations of penetrants to permeate cells, the yield of \( \Delta \)-fructose transformed into \( \Delta \)-soybean sugar was increased 2.1 times.\(^{13}\) Zheng et al. used permeabilized recombinant \( \text{E. coli} \) to produce trehalose, and the maltose conversion rate was increased from 0.6\% to 55.85\% in the control group.\(^{11}\) Oh et al. used cell permeation technology for the first time to realize the production of hydroxy fatty acids in recombinant \( \text{E. coli} \). The conversion yield of 10-hydroxy-12,15(\( \Delta \)-Z)-octadecadienoic acid produced from \( \alpha \)-linolenic acid reached 82\%, which was 17\% higher than that of nonpermeable cells.\(^{16}\) Therefore, permeabilizing the cells is an effective way to increase the substrate conversion rate. According to reports, permeabilization reagents CTAB, DMISO, EDTA, triton X-100, tween-80, and colistin sulfate can effectively change cell permeability and thereby increase the yield of products;\(^{16,23,32-34}\) thus, we explored the effect of these six penetrants on the production of \( \text{trans-2-decenoic acid} \). As shown in Figure 2A,B, triton X-100 and tween-80 could be used as effective penetrants for \( \text{E. coli} \) (\( \Delta \text{fadBJR/fadE-MACS/ydil} \)). At a concentration of 0.5 g/L of the substrate decanoic acid, the conversion rate of \( \text{trans-2-decenoic acid} \) reached 91.9\% and 93.1\%, and the catalytic activity of whole cells was increased by approximately 1.5 times. In contrast, using CTAB, EDTA, colistin sulfate, and DMISO to permeabilize the cells, the transformation rates were only 23.9\%, 55.8\%, 34.6\%, and 39.9\%, respectively (Figures 2C–F). Compared with the 61.9\% conversion rate in the control group, these four permeabilization treatments resulted in a lower conversion rate. This might be because the phospholipid bilayer of the bacterial cell membrane was excessively destroyed in the engineered strain \( \text{E. coli} \) (\( \Delta \text{fadBJR/fadE-MACS/ydil} \)).\(^{35}\)

**Exploring Fatty Acid Production by the Genetically Engineered Strain Producing \( \text{trans-2-Decenoic Acid Inside and Outside the Cell.}** We studied the production of intracellular and extracellular fatty acids under optimal permeabilization conditions. The results in Figure 3 show that when CTAB, colistin sulfate, and DMISO were used as permeabilization reagents, the titers of \( \text{trans-2-decenoic acid} \) both inside and outside the cell were very low. When EDTA was used as a permeabilization reagent, the concentration of extracellular \( \text{trans-2-decenoic acid} \) reached 241.8 mg/L, which was higher than the 220.3 mg/L extracellular concentration in the control group. However, the total amount of \( \text{trans-2-decenoic acid} \) was only 279.1 mg/L, and a downward trend was observed. This might be caused by the excessive permeability of the cell membrane. Triton X-100 and tween-80 as permeabilization reagents can increase the concentration of \( \text{trans-2-decenoic acid} \). These two permeabilization reagents could increase the permeability of cells to a certain extent under the premise of ensuring the integrity of the overall cell structure, which is conducive to the transport of fatty acid molecules and thereby improves the conversion rate of the substrate. When the concentrations of triton X-100 and tween-80 were 2.00\% (v/v) and 1.2\% (v/v), respectively, the detection of extracellular \( \text{trans-2-decenoic acid} \) alone reached 398.8 mg/L and 398.0 mg/L, respectively. The extracellular \( \text{trans-2-decenoic acid} \) content was 5.6 and 5.9 times higher than the intracellular \( \text{trans-2-decenoic acid} \) content. However, in the control group, the extracellular \( \text{trans-2-decenoic acid} \) content was only 2.5 times higher than the intracellular \( \text{trans-2-decenoic acid} \) content. Compared to previous research,\(^{9}\) the yield of \( \text{trans-2-decenoic acid} \) was improved after permeabilization, and the whole-cell catalytic reaction solution obtained in the first step could be directly applied to the second step without disrupting the cells. This made the 10-HDA synthesis procedure more convenient, facilitated the continuous process of the two steps, and further improved the production efficiency.

**Optimization of the Whole-Cell Catalytic Conditions for the Production of \( \text{trans-2-Decenoic Acid Using Engineered Strains.}** The fatty acid concentration is an important effect factor for microbiology.\(^{36}\) It has been reported that increasing the concentration of decanoic acid can alter the composition of cell membranes, thereby affecting the activity of enzymes.\(^{37}\) Therefore, we further investigated the effect of the \( \text{trans-2-decenoic acid} \) conversion rate on different substrate concentrations via permeabilized cells. We found that when the substrate concentration reached 0.8 g/L, the conversion rate of \( \text{trans-2-decenoic acid} \) was only 38.9\% with nonpermeabilized cells. This showed that a high concentration of the substrate tended to lead to low product yield (Figure 4A). After cells were permeabilized with 1.20\% (v/v) tween-80, the conversion rate reached 81.7\%, as the decanoic acid concentration also reached 0.8 g/L (Figure 4B). In terms of increasing the substrate concentration, the cells treated with 2.00\% (v/v) triton X-100 had a better conversion rate. When decanoic acid reached 0.9 g/L, the conversion rate of \( \text{trans-2-decenoic acid} \) reached 86.1\%, and the yield of \( \text{trans-2-decenoic acid} \) was 774.5 mg/L (Figure 4C). We speculated that the permeabilized cells could facilitate the transport of substrates and products, thus resulting in a higher conversion rate compared to that in the intact cell under high decanoic acid concentrations.
In a whole-cell catalysis system, the bacteria are used as the carrier of the catalyst, and the mass concentration of the bacteria greatly affects the speed of the catalytic reaction and the yield of the product.\(^3^8\) Therefore, we explored the effect of cell concentration on the conversion rate of trans-2-decenoic acid. Here, under the premise of controlling the concentration of decanoic acid at 0.9 g/L, we explored the mass concentration gradient effect, induced by the recombinant bacteria, on the product yield, by permeabilizing with 1.20% (v/v) tween-80 and 2.00% (v/v) triton X-100. As a result, the yield of trans-2-decenoic acid increased with an increase in the bacterial cell concentration; however, when the bacterial cell concentration reached 1.75 g/10 mL, the increase in the yield of trans-2-decenoic acid slowed down (Figure 5). This might be because in the high cell concentration system, the viscosity of the reaction solution is high and the oxygen transfer is restricted, making the reaction unable to proceed fully.\(^3^9\) Additionally, a high bacterial cell concentration increases the fermentation cost and is not conducive to large-scale economic fermentation in the later stage.

**Construction and Expression of Fusion Protein CYP153A33/M228L-CPRBM3-GDH and Optimization of Conditions.** The second step of the biocatalytic synthesis of 10-HDA is utilizing P450 enzymes to terminally hydroxylate trans-2-decenoic acid. We obtained a new type of P450 enzyme (CYP153A33/M228L-CPRBM3) through molecular rational design in the early stage. This enzyme was based on the CYP153A33-CPRBM3 fusion protein,\(^4^0\) with a mutation that converted the 228th methionine to leucine.\(^9\) Like that of other P450 enzymes, the catalytic activity of CYP153A33/M228L-CPRBM3 also requires NAD(P)H.\(^2^0,4^1,4^2\) Schewe et al. also mentioned that when the NAD(P)H reserve in the cell is exhausted, P450 oxidation will be limited by the NAD(P)H regeneration rate of the host.\(^2^1\) Therefore, it is an effective and economical strategy to regenerate NAD(P)H by overexpressing the cofactor regenerating enzyme in *E. coli*, thus increasing the availability of cofactors and improving the catalytic performance of P450. We constructed the fusion protein CYP153A33/M228L-CPRBM3-GDH. The GDH gene was from *B. subtilis 168*, which could regenerate the cofactor NAD(P)H by catalyzing the conversion of glucose to gluconolactone.\(^2^4\) We successfully constructed the expression plasmid pET-28a-CYP153A33/M228L-CPRBM3-GDH via seamless cloning (Figure S2A), and the fusion protein was successfully expressed in...
recombinant *E. coli* (CYP153A33/M228L-CPRBM3-GDH) (Figure S2B). To improve the fusion protein expression, the timing of induction, the concentration of IPTG, and the induction time were further optimized. The results showed that when the OD$_{600}$ reached 0.8 and the reaction mixture was induced with 0.5 mM IPTG for 20 h, the concentration of the expressed protein was highest (Figure 6A−C).

**Effect of the Fusion Protein CYP153A33/M228L-CPRBM3-GDH on the Catalysis of trans-2-Decenoic Acid into 10-HDA.** We biosynthesized 10-HDA via recombinant *E. coli* (CYP153A33/M228L-CPRBM3-GDH) using trans-2-decenoic acid as a substrate. In whole-cell catalysis experiments, we controlled the trans-2-decenoic acid concentration range between 0.5 g/L and 1.0 g/L and observed the yield of synthesized 10-HDA by GC-MS (Agilent). We found that when the concentration of trans-2-decenoic acid was 0.7 g/L, the conversion rate was 75.0% and the yield of 10-HDA reached 525.1 mg/L (Figure 6D). When the concentration of trans-2-decenoic acid was >0.8 g/L, the conversion rate decreased rapidly. We inferred that trans-2-decenoic acid had a certain inhibitory effect on the CYP153A33/M228L-CPRBM3-GDH catalytic system.

**Whole-Cell Catalysis of Decanoic Acid to 10-HDA.** In this study, recombinant strains were used to catalyze the conversion of decanoic acid to 10-HDA via a continuous reaction. During the process, the recombinant strain was reacted with 0.9 g/L decanoic acid after permeabilization with 2.00% triton X-100. After a 9 h reaction, according to the GC-MS analysis (Figure S3A), the extracellular yield of trans-2-decenoic acid was 742.1 mg/L. Next, the first step reaction solution supernatant, without breaking the cells, was directly added to recombinant *E. coli* (CYP153A33/M228L-CPRBM3-GDH) for the second whole-cell catalytic step, which simplified the fermentation steps. The results showed that with an increase in the reaction time, the trans-2-decenoic acid produced in the first step of the reaction could be effectively terminaly hydroxylated to produce the final product 10-HDA. After a 20 h reaction (Figure S3B), trans-2-decenoic acid remained at 255.5 mg/L, the yield of 10-HDA reached 486.5 mg/L (Figure 7). We found that conversion rate of the second step was 65.6%, which is lower than using trans-2-decenoic acid as the substrate alone (75−77%). Thus, we speculated that the residual permeabilizing reagent in the first-step reaction solution might influence the second-step whole-cell catalytic reaction. In addition, a small amount of 10-hydroxydecanoic acid (27.5 mg/L) was also detected. This indicates that CYP153A33/M228L-CPRBM3-GDH efficiently

![Figure 6. Optimization of coupled expression conditions of the P450 enzyme and GDH. (A) Effect of the starting induction time on the coupling expression of P450 enzyme and GDH. (B) Effect of the final concentration of the inducer isopropyl-β-D-thiogalactopyranoside on the coupling expression of the P450 enzyme and GDH. (C) Effect of the induction time on the coupling expression of P450 enzyme and GDH. (D) Effect of substrate concentration on the production of 10-hydroxy-2-decenoic acid.](https://doi.org/10.1021/acsomega.2c00972)

![Figure 7. Two-step whole-cell catalysis of decanoic acid to 10-hydroxy-2-decenoic acid.](https://doi.org/10.1021/acsomega.2c00972)
catalyzes the conversion of decanoic acid to 10-hydroxydecanoic acid, and it is difficult for the latter to enter into the β-oxidation step to synthesize 10-HDA.

**CONCLUSION**

In this study, by optimizing the permeating reagent type and concentrations, we found that 2.00% (v/v) triton X-100 had the best effect on increasing the yield of the intermediate product (trans-2-decenoic acid). When decanoic acid reached 0.9 g/L, the conversion rate of trans-2-decenoic acid reached 86.1% and the yield of trans-2-decenoic acid was 77.45 mg/L. Moreover, the extracellular trans-2-decenoic acid content was more than five times the intracellular content. This is the advantage of the two-step synthesis of 10-HDA. The cell suspension of E. coli (CYP153A33/M228L-CPRBM3-GDH) could be directly added to the first-step reaction system consisting of trans-2-decenoic acid, whereas the latter did not require cell fragmentation. E. coli (CYP153A33/M228L-CPRBM3-GDH) could maintain NAD(P)H regeneration during the whole-cell catalysis. Finally, we obtained 486.5 mg/L 10-HDA using decanoic acid as substrate, via the improved two-step whole-cell catalysis, and the two-step conversion rate was 54.1%.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.2c00972.

Additional details and figures (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Jing Su — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China; orcid.org/0000-0002-8604-6629; Phone: 86-531-88631076; Email: sujing@qlu.edu.cn

**Authors**

Li Wang — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Leilei Wang — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Ruiming Wang — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Zhaojun Wang — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Junqing Wang — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Haibo Yuan — State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology, Jinan, Shandong 250353, China; Key Laboratory of Shandong Microbial Engineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong 250353, China

Yan Li — Shandong Freda Biotech Co., Ltd, Jinan, Shandong 250101, China

Suizen Yang — Shandong Freda Biotech Co., Ltd, Jinan, Shandong 250101, China

Tingting Han — Shandong Freda Biotech Co., Ltd, Jinan, Shandong 250101, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00972

**Author Contributions**

∥These authors contributed equally to the work.

**Funding**

This work was supported by the Science Foundation of ShanDong Province (grant number ZR2019MC010), the Synthetic Biology Technology Innovation Center of Shandong Province (grant number dsyntbio-2018-PY-02), the National Science Foundation of China (grant number 31801527), the Focus on Research and Development Plan in Shandong Province [grant numbers 2019]JZZY011003, 2020CXGC010603], and Taishan industry leading talent (grant number tscy2018103).

**Notes**

The authors declare no competing financial interest.

Data Availability: The data for this study are available in the published article and the Supporting Information.

**ACKNOWLEDGMENTS**

The authors are grateful to laboratory master Han Fan (School of Biotechnology, Qilu University of Technology) for providing analytical and technical assistance.

**ABBREVIATIONS**

10-HDA, 10-hydroxy-2-decenoic acid; LB, Luria–Bertani; GDH, glucose dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; CTAB, cetrimonium bromide; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; RND, resistant nodulation cell division family transporter

**REFERENCES**

1. Collazo, N.; Carpena, M.; Nunez-Estevez, B.; Otero, P.; Simal-Gandara, J.; Prieto, M. A. Health Promoting Properties of Bee Royal Jelly: Food of the Queens. *Nutrients* **2021**, *13* (2), 543.

2. Corrana, L.; Biagi, M.; Xiao, J.; Burlando, B. Therapeutic Properties of Bioactive Compounds from Different Honeybee Products. *Front Pharmacol.* **2017**, *8*, 412.
an NADPH-dependent indigo production system. J. Ind. Microbiol. Biotechnol. 2007, 34 (3), 247–53.
(23) Jung, D. H.; Jung, J. H.; Seo, D. H.; Ha, S. J.; Kweon, D. K.; Park, C. S. One-pot bioconversion of sucrose to trehalose using enzymatic sequential reactions in combined cross-linked enzyme aggregates. Bioresearch. Technol. 2013, 130, 801–4.
(24) Zhang, J. D.; Li, A. T.; Yu, H. L.; Imanaka, T.; Xu, J. H. Synthesis of optically pure S-sulfoxide by Escherichia coli transformant cells coexpressing the P450 monooxygenase and glucose dehydrogenase genes. J. Ind. Microbiol Biotechnol. 2011, 38 (5), 633–41.
(25) Zhang, J.; Li, X. Novel strategy for phenylactic acid biosynthesis from phenylalanine by whole cell recombinant Escherichia coli coexpressing γ-phenylalanine oxidase and γ-lactate dehydrogenase. Biotechnol. Lett. 2018, 40 (1), 165–171.
(26) Zhou, Y.; Lu, Z.; Wang, X.; Selvaraj, J. N.; Zhang, G. Genetic engineering modification and fermentation optimization for extracelluar production of recombinant proteins using Escherichia coli. Appl. Microbiol. Biotechnol. 2018, 102 (4), 1545–1556.
(27) Wu, J.; Wang, Z.; Zhang, X.; Zhou, P.; Xia, X.; Dong, M. Improving medium chain fatty acid production in Escherichia coli by multiple transporter engineering. Food Chem. 2019, 272, 628–634.
(28) van den Berg, B.; Black, P. N.; Clemons, W. M.; Rapoport, T. A. Crystal structure of the long-chain fatty acid transporter FadL. Science. 2004, 304, 1506–1509.
(29) Jeon, E. Y.; Song, J. W.; Cha, H. J.; Lee, S. M.; Lee, J.; Park, J. B. Intracellular transformation rates of fatty acids are influenced by expression of the fatty acid transporter FadL in Escherichia coli cell membrane. J. Biotechnol. 2018, 281, 161–167.
(30) Lepore, B. W.; Indic, M.; Pham, H.; Hearn, E. M.; Patel, D. R.; van den Berg, B. Ligand-gated diffusion across the bacterial outer membrane. Proc. Natl. Acad. Sci. U. S. A. 2011, 108 (25), 10121–6.
(31) Zheng, Z.; Xu, Y.; Sun, Y.; Mei, W.; Ouyang, J. Biocatalytic Production of Trehalose from Maltose by Using Whole Cells of Permeabilized Recombinant Escherichia coli. PLoS One. 2015, 10 (10), No. e0140477.
(32) Cortes, D. V.; Roberto, I. C. CTAB, Triton X-100 and freezing-thawing treatments of Candida guilliermondii: effects on permeability and accessibility of the glucose-6-phosphate dehydrogenase, xylitol reductase and xylitol dehydrogenase enzymes. N Biotechnol. 2012, 29 (2), 192–8.
(33) Li, J. Y.; Zhang, L. W.; Du, M.; Han, X.; Yi, H. X.; Guo, C. F.; Zhang, Y. C.; Luo, X.; Zhang, Y. H.; Shan, Y. J.; Hou, A. J. Effect of tween series on growth and cis-9, trans-11 conjugated linoleic acid production of Lactobacillus acidophilus FO221 in the presence of bile salts. Int. J. Mol. Sci. 2011, 12 (12), 9138–54.
(34) Wang, G. L.; Din, A. U.; Qi, Y. S.; Wang, C. L.; Wang, D. H.; Wei, G. Y. Triton X-100 improves co-production of β-1,3-glucan and pullulan by Aureobasidium pullulans. Appl. Microbiol. Biotechnol. 2020, 104 (24), 10685–10696.
(35) Krauser, S.; Weyer, C.; Bläss, L. K.; Heinzle, E. Directed Multistep Biocatalysis Using Tailored Permeabilized Cells. Fundamentals and Application of New Bioproduction Systems 2013, 137, 185–234.
(36) Skrivanova, E.; Marounek, M.; Dlouha, G.; Kanka, J. Susceptibility of Clostridium perfringens to C2-C18 fatty acids. Lett. Appl. Microbiol. 2005, 41 (1), 77–81.
(37) Alexandre, H.; Mathieu, B.; Charpentier, C. Alteration in membrane fluidity and lipid. Microbiology. 1996, 142, 469–475.
(38) Chen, J. P.; Lin, G. H. Optimization of biodiesel production catalyzed by fungus cells immobilized in fibrous supports. Appl. Biochem. Biotechnol. 2010, 161 (1–8), 181–94.
(39) Garcia-Ochoa, F.; Gomez, E. Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnol. Adv. 2009, 27 (2), 153–76.
(40) Notonier, S.; Griman, L.; Pleiss, J.; Hauer, B. Semipreparative Protein Engineering of CYPI5A1Δag -CPRBM3 for Efficient Terminal Hydroxylation of Short- to Long-Chain Fatty Acids. Chembiochem. 2016, 17 (16), 1550–7.
(41) Lundemo, M. T.; Notonier, S.; Striedner, G.; Hauer, B.; Woodley, J. M. Process limitations of a whole-cell P450 catalyzed reaction using a CYP153A-CPR fusion construct expressed in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2016**, **100** (3), 1197–1208.

(42) Zhao, C.; Song, G.; Silver, K.; Tang, T.; Wang, C.; Qiu, L. Heterologous Co-expression of CYP6B7 and NADPH-Dependent Cytochrome P450 Reductase From *Helicoverpa armigera* (Lepidoptera: Noctuidae) in *Pichia pastoris*. *J. Econ Entomol.* **2018**, **111** (4), 1868–1874.