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

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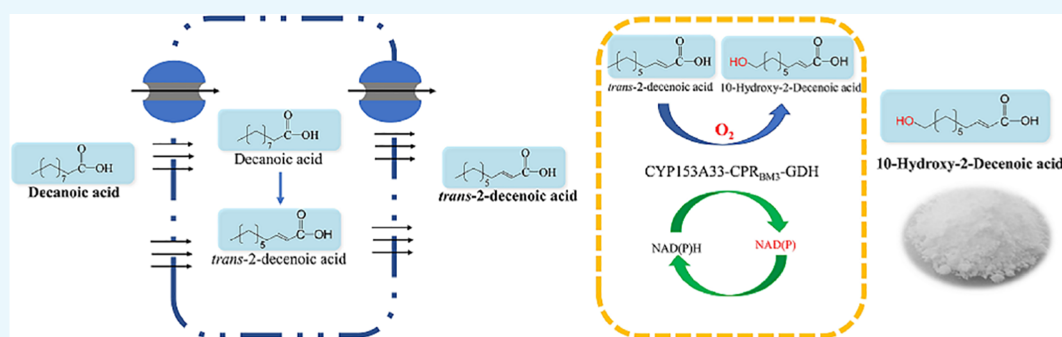

ABSTRACT: 10-Hydroxy-2-decenoic acid (10-HDA) is an α,β-unsaturated medium-chain carboxylic acid containing a terminal hydroxyl group. It has various unique properties and great economic value. We improved the two-step biosynthesis method of 10-HDA. The conversion rate of the intermediate product *trans*-2-decenoic 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-CPR$_{BM3}$) 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 α,β-unsaturated carboxylic acid derivatives.

■ INTRODUCTION

10-Hydroxy-2-decenoic acid (10-HDA) is a terminal hydroxylated medium-chain α,β-unsaturated carboxylic acid. This chemical performs many unique physiological activities,[1,2] 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.[3,4] The traditional physical extraction method is associated with high production costs, thus resulting in failure to meet the market demand.[5] 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.[6,7] 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.[8] Recently, the biosynthesis of 10-HDA was reported using decanoic acid as a substrate via two-step whole-cell catalysis.[9] In the first step, decanoic acid was converted to *trans*-2-decenoic acid through the modified β-oxidation pathway in *Escherichia coli*. In the second step, CYP153A33/M228L-CPR$_{BM3}$ 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

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Table 1. List of Primers

primer names	primer base sequence (5′ → 3′)
Pf_fadL(XhoI)	gctgacgtcggtaccctcgagATGAGCCAGAAAACCCTGTTTACA
Pr_fadL(PacI)	tggcagcagcctaggttaattaaTCAGAACGCGTAGTTAAAGTTAGTACC
Pf_sumo-^{Ct}ydiI(*Bam*HI)	tcatcaccacagccaggatccATGTCGGACTCAGAAGTCAATCAA
Pr_sumo-^{Ct}ydiI(*Hin*dIII)	gcattatgcggccgcaagcttCTCGAGTTACACAACGGCGG
Pf_mdtE(NdeI)	taagaaggagatatacatatgATGAACAGAAGAAGAAAGCTGTTAATACC
Pr_mdtE(XhoI)	ggtttctttaccagactcgagTTATTGTTTCGATTCGGTGCTG
Pf_acrE(NcoI)	taagaaggagatataccatggATGACGAAACATGCCAGGTTTT
Pr_acrE(*Bam*HI)	gccgagctcgaattcggatccTTACTTCGATGCAGTATCTGCGG
Pf_sumo-^{Ct}ydiI(AscI)	tccgaattcgagctcggcgcgccATGTCGGACTCAGAAGTCAATCAA
Pr_sumo-^{Ct}ydiI(*Hin*dIII)	gcattatgcggccgcaagcttCTCGAGTTACACAACGGCGG
Pf_CYP153(NdeI)	gtgccgcgcggcagccatatgATGCCGACGTTACCACGTACC
Pr_ CYP153	aatttacccatCCCAGCCCACACGTCTTTT
Pf_GDH	tgggctgggATGGGTAAATTTGAAGGTAAAATAGCG
Pr_GDH(XhoI)	gtggtggtggtggtgctcgagTTATACCTGTGCTAGCCCGCC

Table 2. List of Engineering Strain and Plasmid Names

strain/plasmid	description	source (ref)
E. coli BL21(DE3) (Δ*fadBJR*)	Δ*fadB*::FRT Δ*fadJ*::FRT Δ*fadR*::FRT sequential deletion of *fadB*, *fadJ*, and *fadR* harboring a λDE3 lysogen	9
pCDFDuet-1-^{Pp}*fadE*-^{Ma}*MACS*	pCDFDuet-1 Carry ^{Pp}*fadE* and ^{Ma}*MACS* gene	9
pET-28a-sumo-^{Ct}*ydiI*	pET-28a Carry ^{Ct}*ydiI* gene	9
pET-21b-*CYP153A33/M228L-CPR*$_{BM3}$	pET-21b Carry *CYP153A33/M228L-CPR*$_{BM3}$ gene	9
pETDuet-1-sumo-^{Ct}*ydiI-fadL*,	pETDuet-1 Carry ^{Ct}*ydiI* and *fadL* gene	this study
pETDuet-1-*acrE*-sumo-^{Ct}*ydiI-mdtE*	pETDuet-1 Carry *acrE* and ^{Ct}*ydiI* and *mdtE* gene	this study
pET-28a-*CYP153A33/M228L-CPR*$_{BM3}$-*GDH*	pET-28a Carry *CYP153A33/M228L-CPR*$_{BM3}$ and *GDH* gene	this study
E. coli (Δ*fadBJR/fadE-MACS/ydiI*)	*E. coli* BL21(DE3) (Δ*fadBJR*/ pCDFDuet-1-^{Pp}*fadE*-^{Ma}*MACS*/ pET-28a-sumo-^{Ct}*ydiI*)	9
E. coli (*CYP153A33/M228L-CPR*$_{BM3}$)	*E. coli* BL21(DE3) (pET-21b-*CYP153A33/M228L-CPR*$_{BM3}$)	9
E. coli (*CYP153A33/M228L-CPR*$_{BM3}$-*GDH*)	*E. coli* BL21(DE3) (pET-28a-*CYP153A33/M228L-CPR*$_{BM3}$-*GDH*)	this study

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. Lennen et al. showed that AcrAB-TolC in *E. coli*, localized across the cell membrane, can transport most of the produced fatty acids outside the cell and improve their titer.[12] Moreover, cell permeability can reduce the mass transfer resistance, reduce the toxic effect on cells, and thereby increase the production of metabolites.[13−15] Oh et al. used cell permeation technology for the first time to realize the production of hydroxy fatty acids in recombinant *E. coli*. The conversion yield of 10-hydroxy-12,15(*Z*,*Z*)-octadecadienoic acid produced from α-linolenic acid reached 82%, which was 17% higher than that with nonpermeable cells.[16]

The second step in the synthesis of 10-HDA is the terminal hydroxylation of *trans*-2-decenoic acid. We obtained a new type of P450 enzyme (CYP153A33/M228L-CPR$_{BM3}$) through molecular rational design during the initial study, which has high catalytic activity on *trans*-2-decenoic acid.[9] CYP153A33/M228L-CPR$_{BM3}$ is a self-sufficient P450 enzyme composed of CYP153A33/M228L and CPR$_{BM3}$, the latter being the reducing domain of CYP102A from *Bacillus megaterium*. The CYP102 family, CYP116 family, and CYP505 family are all self-sufficient P450 enzymes in nature, which encode their heme domain and reductase protein in a single polypeptide. The self-sufficient P450 has a high conversion rate and is easily used in biotechnological applications.[17−19] However, the CYP153A33/M228L-CPR$_{BM3}$ enzyme requires NAD(P)H, like all the P450 enzymes, and its catalytic activity will be limited by the NAD(P)H regeneration rate of the host.[20,21] 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.[22−24]

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$_{BM3}$) with glucose dehydrogenase (GDH) derived from *Bacillus subtilis 168* (*B. subtilis 168*), which increased the reaction efficiency of the conversion from *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.

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■ 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-^{Pp}*fadE*-^{Ma}*MACS*, pET-28a-sumo-^{Ct}*ydiI*, and pET-21b-*CYP153A33/M228L-CPR*$_{BM3}$ were all constructed as mentioned previously.[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-^{Ct}*ydiI-fadL*, pETDuet-1-sumo-*acrE*-sumo-^{Ct}*ydiI-mdtE*, and pET-28a-*CYP153A33/M228L-CPR*$_{BM3}$*-GDH* were successfully constructed. The plasmids pETDuet-1-sumo-^{Ct}*ydiI-fadL* and pETDuet-1-sumo-*acrE*-sumo-^{Ct}*ydiI-mdtE* yielded the coexpression of thioesterase (^{Ct}YdiI) with different fatty acid transporters. The plasmid pET-28a-*CYP153A33/M228L-CPR*$_{BM3}$*-GDH* comprised coexpression of the P450 enzyme (CYP153A33/M228L-CPR$_{BM3}$) 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) (Δ*fadBJR*) was used as a starting strain for the synthesis *trans*-2-decenoic acid from decanoic acid, which comprised the deletion of three genes (*fadB*, *fadJ*, and *fadR*) involved in β-oxidation.[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 μg/mL), kanamycin (50 μg/mL), and ampicillin (100 μg/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-β-D-thiogalactopyranoside (IPTG; 0.5 mM) was added to the culture medium, and the engineered strains were subjected to continued incubation for 20 h. In *E. coli* (*CYP153A33/M228L-CPR*$_{BM3}$) and *E. coli* (*CYP153A33/M228L-CPR*$_{BM3}$*-GDH*), 0.5 mM 5-aminolevulinic acid hydrochloride and 0.5 mM $FeCl_3$ 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 15 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 cetrimonium 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 μg/mL streptomycin, and 50 μg/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-CPR$_{BM3}$-GDH Expression and Whole-Cell Catalysis System of *trans*-2-Decenoic Acid to 10-HDA. The induction conditions of *E. coli* (*CYP153A33/M228L-CPR*$_{BM3}$*-GDH*) are important and have an important effect on the expression of heterologous proteins.[22,25,26] 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-CPR$_{BM3}$-GDH was analyzed. The whole-cell catalysis system of *E. coli* (*CYP153A33/M228L-CPR*$_{BM3}$*-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 μg/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.[9] The whole-cell catalysis of decanoic acid to produce 10-HDA was performed as follows: The cultured recombinant bacteria *E. coli* (Δ*fadBJR/fadE-MACS/ydiI*) 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-CPR*$_{BM3}$*-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 μM $FeSO_4$, and kanamycin (50 μg/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).[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) (Δ*fadBJR*), consisting of two different plasmids. The first plasmid, pCDFDuet-1-^{Pp}*fadE*-^{Ma}*MACS*, was derived from our previous research,[9] and the second plasmid was used to coexpress the thioesterase gene ^{Ct}*ydiI* and the transporter gene (Figure S1). Here, we investigated two types of transporters. The first was the combination AcrE and MdtE transporters, which were

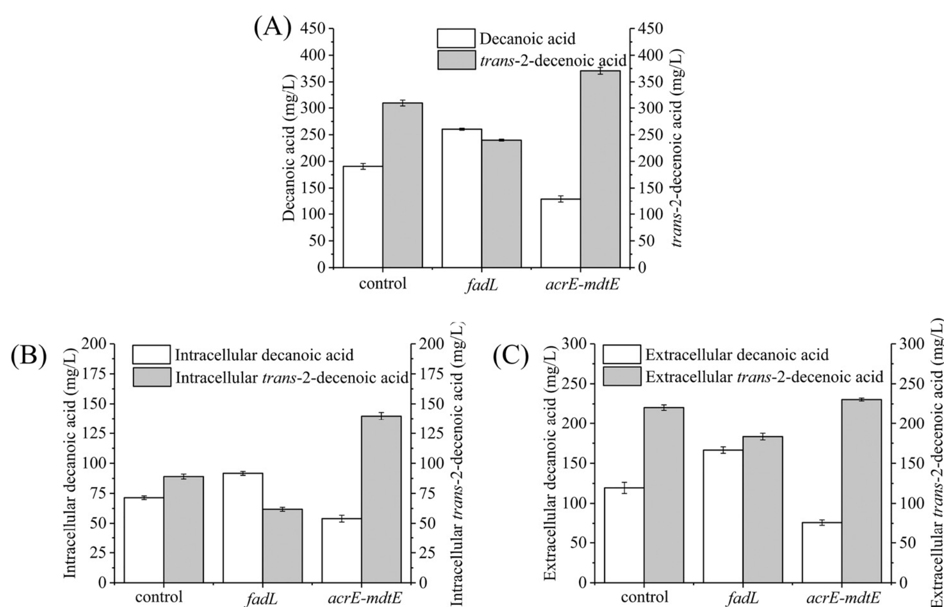

Figure 1. Effect of transporters on the production of *trans*-2-decenoic acid. (A) Effect of resistant nodulation cell division family transporter (RND) and outer membrane protein on the production of total *trans*-2-decenoic acid. (B) Effect of RND family and outer membrane protein on the production 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* (Δ*fadBJR*/*fadE-MACS*/*ydiI*) to study the effect of transporter engineering on medium-chain fatty acid production.

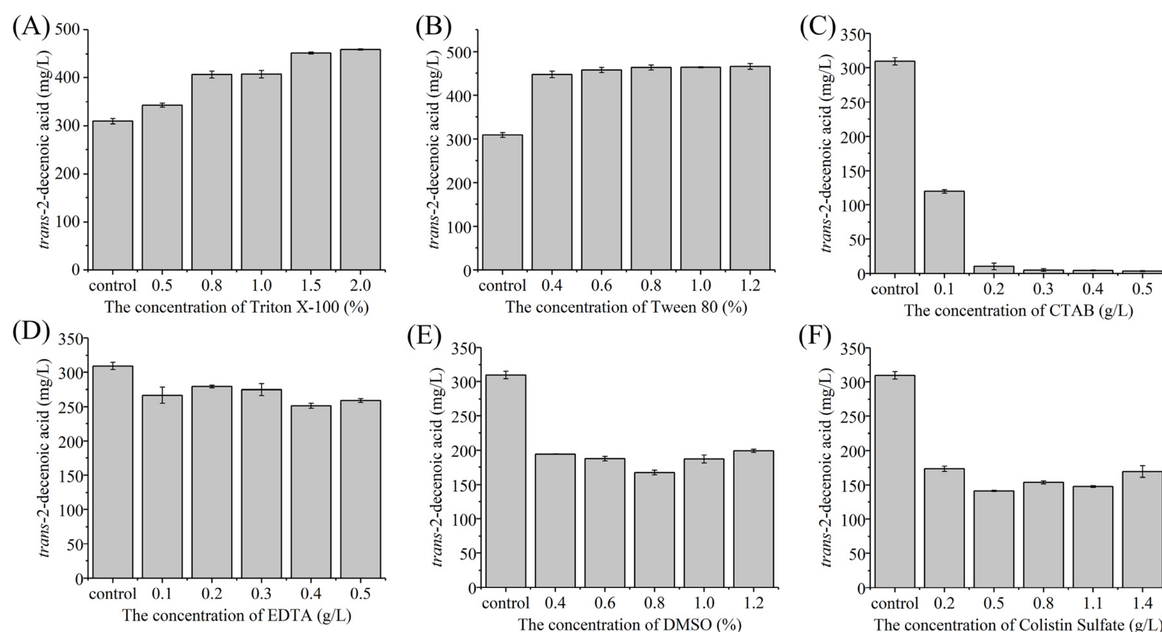

Figure 2. Effect of different permeabilization reagents on the engineered strain producing *trans*-2-decenoic acid. (A) Triton X-100. (B) Tween-80. (C) CTAB. (D) EDTA. (E) DMSO. (F) Colistin sulfate.

derived from the resistance-nodulation cell division (RND) superfamily.[12] They were used for improving the yield of medium-chain fatty acids (C_6–C_{10}).[27] 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.[28,29] Finally, we constructed endogenous transporter recombinant plasmids, namely pETDuet-1-sumo-^{Ct}*ydiI-fadL* and pETDuet-1-*acrE*-sumo-^{Ct}*ydiI-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 increase 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,[27] 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 *trans*-

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2-decenoic acid transport. Additionally, expression of the cell outer membrane protein FadL reduced the total concentration of *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 (C_{12}−C_{18}).[30]

Comparison of Different Permeabilization Treatments Applied to *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 D-fructose transformed into D-soybean sugar was increased 2.1 times.[13] Zheng et al. used permeabilized recombinant *E. coli* to produce trehalose, and the maltose conversion rate was increased from 0.6% to 55.85% in the control group.[31] Oh et al. used cell permeation technology for the first time to realize the production of hydroxy fatty acids in recombinant *E. coli*. The conversion yield of 10-hydroxy-12,15(*Z*,*Z*)-octadecadienoic acid produced from α-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, DMSO, 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 *trans*-2-decenoic acid. As shown in Figure 2A,B, triton X-100 and tween-80 could be used as effective penetrants for *E. coli* (Δ*fadBJR*/*fadE*-*MACS*/*ydiI*). At a concentration of 0.5 g/L of the substrate decanoic acid, the conversion rate of *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 DMSO 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 *E. coli* (Δ*fadBJR*/*fadE*-*MACS*/*ydiI*).[35]

Exploring Fatty Acid Production by the Genetically Engineered Strain Producing *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 DMSO were used as permeabilization reagents, the titers of *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 *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 *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 *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 2.0% (v/v) and 1.2% (v/v), respectively, the detection of extracellular *trans*-2-decenoic acid alone reached 398.8 mg/L and 398.0 mg/L, respectively. The extracellular *trans*-2-decenoic acid content was 5.6 and 5.9 times higher than the intracellular *trans*-2-decenoic acid content. However, in the control group, the extracellular *trans*-2-decenoic acid content was only 2.5 times higher than the intracellular *trans*-2-decenoic acid content. Compared to previous research,[9] the yield of *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.

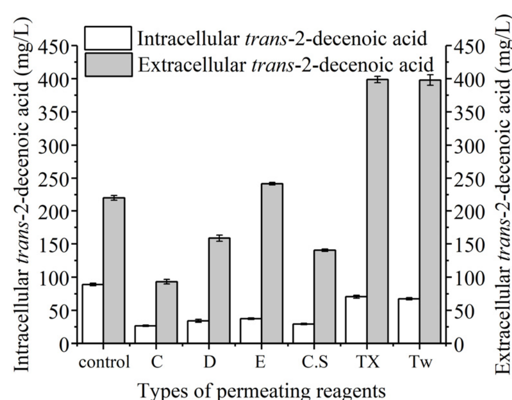

Figure 3. Distribution of fatty acids inside and outside the cells of engineered strain that produce *trans*-2-decenoic acid at the optimal permeabilization reagent treatment concentration. C, CTAB; D, DMSO; E, EDTA; C.S, colistin sulfate; TX, Triton X-100; Tw, tween-80.

Optimization of the Whole-Cell Catalytic Conditions for the Production of *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 *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 *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 *trans*-2-decenoic acid reached 86.1%, and the yield of *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 concentration rate compared to that in the intact cell under high decanoic acid concentrations.

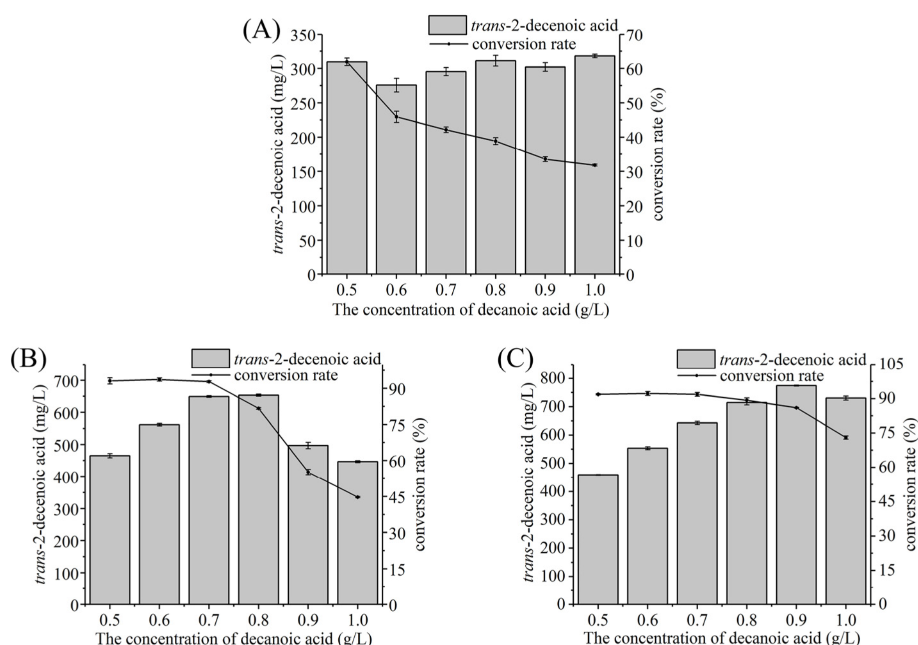

Figure 4. Effect of substrate concentration on the production of *trans*-2-decenoic acid under different treatment conditions. (A) Control. (B) Tween-80. (C) Triton X-100. The range of substrate concentration was 0.5−1.0 g/L.

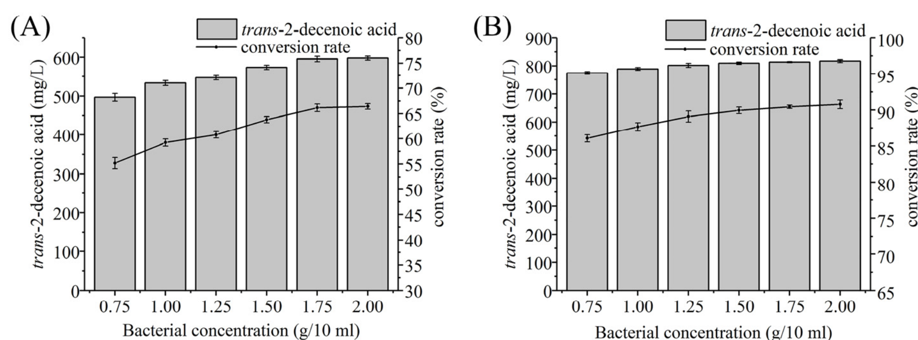

Figure 5. Effect of bacterial concentration on the production of *trans*-2-decenoic acid under the conditions of permeabilization treatment. (A) Tween-80. (B) Triton X-100. The range of the bacterial concentration was 0.75−2.00 g/10 mL.

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.[38] 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.[39] 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-CPR$_{BM3}$-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-CPR$_{BM3}$) through molecular rational design in the early stage. This enzyme was based on the CYP153A33-CPR$_{BM3}$ fusion protein,[40] with a mutation that converted the 228th methionine to leucine.[9] Like that of other P450 enzymes, the catalytic activity of CYP153A33/M228L-CPR$_{BM3}$ also requires NAD(P)H.[20,41,42] 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.[21] 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-CPR$_{BM3}$-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.[24] We successfully constructed the expression plasmid pET-28a-*CYP153A33/M228L-CPR$_{BM3}$-GDH* via seamless cloning (Figure S2A), and the fusion protein was successfully expressed in

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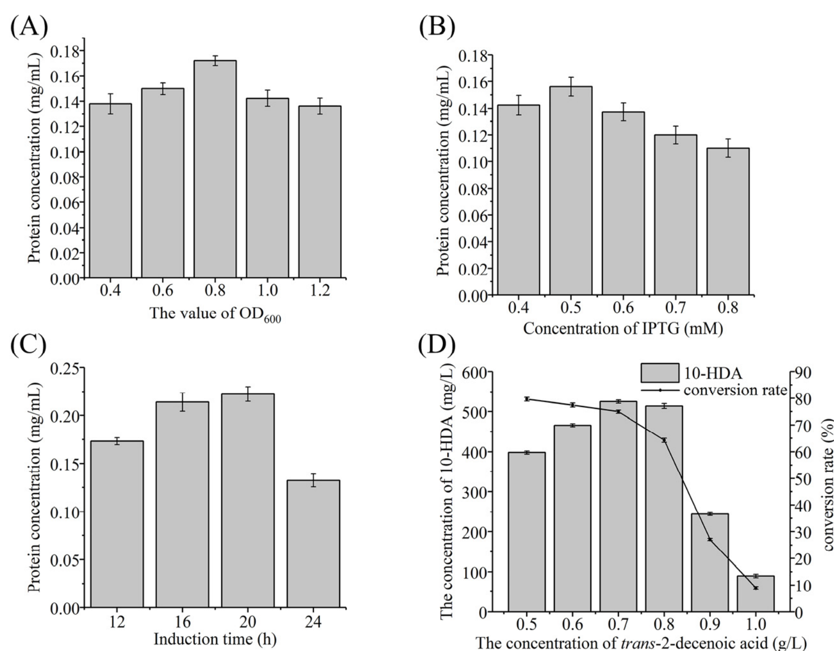

Figure 6. Optimization of coupled expression conditions of the P450 enzyme and GDH. (A) Effect of the starting induction time on the coupling expression of P450 enzyme and GDH. (B) Effect of the final concentration of the inducer isopropyl-β-D-thiogalactopyranoside on the coupling expression of the P450 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.

recombinant *E. coli* (*CYP153A33/M228L-CPR$_{BM3}$-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-CPR$_{BM3}$-GDH* on the Catalysis of *trans*-2-Decenoic Acid into 10-HDA. We biosynthesized 10-HDA via recombinant *E. coli* (*CYP153A33/M228L-CPR$_{BM3}$-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-CPR$_{BM3}$-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-CPR$_{BM3}$-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 terminally 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

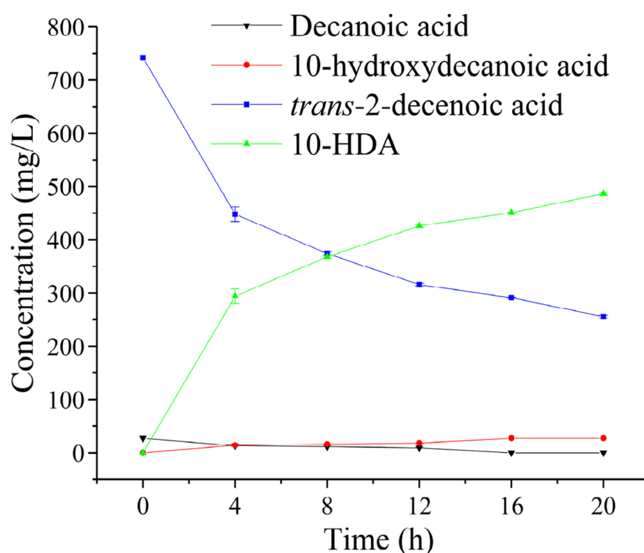

Figure 7. Two-step whole-cell catalysis of decanoic acid to 10-hydroxy-2-decenoic acid.

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-CPR$_{BM3}$-GDH efficiently

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 774.5 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-CPR*$_{BM3}$*-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-CPR*$_{BM3}$*-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/doi/10.1021/acsomega.2c00972.

Additional details and figures (PDF)

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Notes

The authors declare no competing financial interest.

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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

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