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Enhancement of α,ω-Dicarboxylic Acid Production by the Expression of Xylose Reductase for Refactoring Redox Cofactor Regeneration

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Supporting Information

ABSTRACT: The production of α,ω-dicarboxylic acids (DCAs) by whole-cell biocatalysis is often limited by cofactor regeneration. Here, ω-oxidation pathway genes (monooxygenase, alcohol dehydrogenase, and aldehyde dehydrogenase) were coexpressed with a xylose reductase (XR) gene to regenerate cofactors in an engineered *Escherichia coli* strain that cometabolizes glucose and xylose. The resulting strain exhibited a 180% increase in DCA production compared with the control strain without XR, and produced xylitol in the presence of xylose. Expression of monooxygenase and XR without other ω-oxidation pathway genes resulted in an additional increase in tetradecanedioic acid concentration and a substrate conversion of 95%, which was 198% higher than that associated with the control strain. The expression of XR helped the system to regenerate and balance the cofactors thereby achieving maximum substrate conversion efficiency. It could serve as an efficient platform for the industrial production of α,ω-DCAs.

KEYWORDS: *α,ω-dicarboxylic acids, cofactor regeneration, CYP450s, fatty acids, redox balance, ω-oxidation pathway*

INTRODUCTION

Terminally ω-oxyfunctionalized carboxylic acids (α,ω-dicarboxylic acids, DCAs), generally represented as HOOC-$(R)_n$-COOH, are multifunctional organic compounds used as building blocks for polyamides, polyurethanes, and polyesters.[1,2] Furthermore, DCAs have direct commercial utility as perfumes, adhesives, antiseptics, lubricants, and therapeutics.[1,3] The conventional chemical conversion process for the production of DCAs from petrochemicals suffers from several environmental issues and limitations.[4,5] Moreover, fatty acids (FAs), being abundant renewable resources found as plant oils and animal fats, are considered attractive substrates for the production of various value-added chemicals, including DCAs.[6−8] However, the terminal oxyfunctionalization of FAs by chemocatalysis is not widely practiced because of several limitations associated with this process: energetically demanding and harsh reaction conditions, generation of numerous byproducts, and the difficulty of achieving selectivity and regiospecificity.[9,10]

Whole-cell biotransformation is preferable for the production of DCAs for various reasons including cost effectiveness, increased biocatalyst stability, simultaneous regeneration of cofactors through cell metabolism, and high product concentrations.[11,12] The biological process for the production of DCAs, which proceeds via cytochrome P450 (CYP450) monooxygenase-catalyzed terminal oxyfunctionalization of FAs, using molecular oxygen as a green oxidant, and oxidation of the resulting hydroxy-FAs to DCAs, could be a greener and more cost-efficient alternative to chemical processes.[5,7,13−16] However, several factors, including substrate and oxygen accessibility, toxicity and process inhibition associated with the substrate and product, and the ability of the cells to regenerate cofactors, affect the efficiency of whole-cell CYP450-catalyzed reactions.[7,17,18]

In a previous study, we engineered an *Escherichia coli* strain to produce long-chain α,ω-DCAs [dodecanedioic acid (DDDA) and tetradecanedioic acid (TDDA)] from FAs by expression of a heterologous ω-oxidation pathway, starting with a CYP450-catalyzed reaction.[7] In this pathway, the methyl group at the ω-carbon of a FA is first oxidized to a hydroxyl group by a fusion protein composed of the monooxygenase CYP153A from *Marinobacter aquaeolei* and the NADPH:cytochrome P450 oxidoreductase domain of CYP102A1 from *Bacillus megaterium*. The resulting hydroxyl group is then oxidized to an oxo group by a NAD-dependent alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae*, and finally to a carboxyl group by a NAD-dependent aldehyde dehydrogenase (ALD) from *Candida tropicalis*, thereby producing α,ω-DCAs. However, the resulting engineered *E. coli* strain (REω) showed low substrate conversion efficiencies of approximately 14% and 36% with C12- and C14-FAs, respectively.[7] It is speculated that the ω-oxidation pathway suffers from redox cofactor imbalance; the pathway results in oxidation of one NADPH molecule and reduction of two $NAD(P)^+$ molecules upon conversion of a FA to a DCA. Consequently, one oxidized form of $NADP^+$ molecule and two reduced form of NAD(P)H molecules produced by the ω-oxidation pathway must be regenerated, causing redox balance perturbation. The overall performance of whole-cell biotransformation is often limited by redox cofactor imbalance[19,20] because of the limitations associated with cofactor generation using cellular metabolism alone.[21]

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Table 1. Strains and Plasmids Used in This Study

strains and plasmids	genotype and description	reference
Strains		
REω	*E. coli* MG1655 with Δ*fadE*::FRT, Δ*fadR*::FRT harboring pBbA6c-ω	7
GX50Δ*xylAB*	*E. coli* MG1655 with Δ*araC*::FRT, Δ*xylA*::FRT, Δ*xylB*::FRT, P_{CP25}-*araB*, P_{CP6}-*araF*, P_{CP6}-*araE*, P_{CP25}-*xylA*, P_{CP6}-*xylF*. Deletion and replacement mutations in noncoding regions upstream of *pyrE* and *xylA*, and the point mutations S91I and D99G in *araE* and *ybjG*, respectively (here simply referred to as GX).	26
GXER	GX with Δ*fadE*::FRT, Δ*fadR*::FRT	this study
GXERC	GXER with Δ*puuC*::FRT	this study
GXERω	GXER harboring pBbA6c-ω	this study
GXERω-XR_{NADPH}	GXERω harboring pXR_{NADPH}	this study
GXERω-XR_{NADH}	GXERω harboring pXR_{NADH}	this study
GXERC-M	GXERC harboring pA6c-FP/AlkL	this study
GXERC-MXR_{NADPH}	GXERC harboring pA6c-FP/AlkL and pXR_{NADPH}	this study
GXER-M	GXER harboring pA6c-FP/AlkL	this study
GXER-MXR_{NADPH}	GXER harboring pA6c-FP/AlkL and pXR_{NADPH}	this study
Plasmids		
pBbA6c-ω	pBbA6c with Δ*rfp*::CYP450_NCP/AlkL/ADH/ALD, Cm^R	7
pA6c-FP	pBbA6c with Δ*rfp*::CYP450_NCP, Cm^R	7
pA6c-FP/AlkL	pBbA6c with Δ*rfp*::CYP450_NCP/AlkL, Cm^R	7
pE6k-ADH/ALD	pBbE6k with Δ*rfp*::ADH/ALD, Km^R	7
pXR_{NADPH}	pBbB6a with Δ*gfp*::*xyl1* (*Candida boidinii*, NADPH dependent xylose reductase), Am^R	26
pXR_{NADH}	pBbB6a with Δ*gfp*::$xyl1_m$ (Lys^{272}, Ser^{273}, and Asn^{274} to Arg, Glu, and Gly; NADH-dependent xylose reductase), Am^R	this study

Various strategies to overcome the limitations of cofactor regeneration in whole-cell biotransformation systems have been reported, including external addition of synthetic cofactors in stoichiometric quantities, as well as chemical, photochemical, and electrochemical methods.[22] However, these methods suffer from certain disadvantages, such as the high cost of cofactors, the requirement for high overpotentials, electrode fouling, and the requirement for expensive chemical mediators.[23,24] Thus, biological methods for cofactor regeneration mediated by enzymes (*in vitro*, using purified enzymes) or whole cells (*in vivo*, using whole cells expressing enzymes for cofactor regeneration) are potential alternatives. We therefore hypothesized that the use of a biological cofactor-regenerating system would enhance the efficiency of α,ω-DCA production in our system.[7,11,18,25] Recently, Kim and co-workers developed an *E. coli* strain (GX50Δ*xylAB*, hereafter referred to as GX) that can coutilize glucose and xylose and produce a sugar alcohol, xylitol, using an NADPH-dependent xylose reductase (XR).[26] This system can be used for cofactor regeneration. XR, an oxidoreductase (E.C. 1.1.1.307), is responsible for converting aldoses into the corresponding polyols and *vice versa*, using pyridine nucleotides as cosubstrates.[27,28] XR is widely employed for ethanol and xylitol production in various bacteria and yeast.[26,29,30]

In the present study, we report the enhanced production of DCAs in a GX variant with additional genome modifications by employing XR to regenerate cofactors. To the best of our knowledge, this is the first report on the use of XR to enhance the production of α,ω-DCAs from FAs.

MATERIALS AND METHODS

Microbial Strains and Plasmids. An *E. coli* MG1655 strain, GX (originally referred to as GX50Δ*xylAB*),[26] was used as the parental strain for all genetic modifications including gene knockouts and introduction of expression constructs. *E. coli* strain DH10B (laboratory stock) was used for cloning. The constructed strains and plasmids are listed in Table 1.

Chemicals, Enzymes, and Culture Conditions. All chemicals, including FAs and DCAs, were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes (New England Biolabs, Ipswich, MA), DNA ligase (New England Biolabs, Ipswich, MA), and Phusion high-fidelity DNA polymerase (Thermo Fischer Scientific, Waltham, MA) were used for cloning and plasmid construction. Microbial cells were grown at 37 °C in Luria−Bertani medium with continuous shaking (200 rpm). The media were supplemented with suitable antibiotics where required at the following concentrations: kanamycin (Km) 50 μg/mL, ampicillin (Am) 100 μg/mL, and chloramphenicol (Cm) 30 μg/mL.

Strain and Plasmid Construction. Genes, *fadE*, *fadR*, and *puuC*, were knocked out in strain GX using a Lambda Red and FLP-mediated site-specific recombination system, as described previously.[31] Construction of all plasmids was carried out according to standard protocols.[32] Primers used in this study are listed in Table S1. The constructed plasmids were introduced into the relevant strains by electroporation using a MicroPulser electroporator (Bio-Rad). Site-directed mutations in XR (XR_{NADPH}) were introduced by PCR with mutagenic primers, and the resulting gene fragments were assembled by overlap extension PCR. The cofactor dependency of XR was altered from NADPH to NADH by targeting residues Lys^{272}, Ser^{273}, and Asn^{74} in the NADPH binding pocket of XR. These residues were changed to Arg, Glu, and Gly, respectively, yielding a greater than 10^4-fold change in specificity for NADH.[27] All constructs were confirmed by DNA sequencing (Macrogen, Korea).

Whole-Cell Biotransformation. Whole-cell biotransformations using the engineered *E. coli* strains were performed as described previously.[7] Briefly, resting cells were prepared by growth of each strain in Luria−Bertani media to an OD_{600} of 0.5−0.6, addition of 0.1 mM IPTG to induce expression of target genes, together with 0.25 mM 5-aminolevulinic acid as a heme precursor and subsequent incubation of the cells with shaking at 200 rpm and 25 °C for 20 h to facilitate soluble expression of the target genes. The recombinant cells were harvested by centrifugation at 12 000 × *g* for 15 min at 4 °C, washed twice with potassium phosphate (KPi) buffer (0.1 M, pH 7.4), and used as biocatalysts in the biotransformation studies. Biotransformation was conducted in 250 mL Erlenmeyer shaking flasks by adding a 20% FA (C12 or C14) substrate stock solution in DMSO into KPi buffer (10 mL) containing 50 g_{cww}/L of the induced resting cells, 0.5% Tween 80, 0.1 mM thiourea, and 1× trace element solution (per L: 2.4 g $FeCl_3 \cdot 6H_2O$, 0.3 g $CoCl_2 \cdot H_2O$, 0.15 g $CuCl_2 \cdot 2H_2O$, 0.3 g $ZnCl_2$, 0.3 g $Na_2MO_4 \cdot 2H_2O$, 0.075 g H_3BO_3, and 0.495 g $MnCl_2 \cdot 4H_2O$).

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Finally, the ability of strain GXER-MXR$_{NADPH}$ to produce DCAs from FAs (1 g/L C12 or C14) was examined. All biotransformations were conducted as described above; the reaction mixtures contained cosubstrates [0.4% (*w*/*v*) each of D-glucose (Glu) and D-xylose (Xyl)] and were incubated at 30 °C in a shaking incubator (250 rpm). Samples were collected at different time points for quantification of DCAs, sugars (Glu and Xyl), xylitol, and NADPH/NADP$^+$.

Analytical and Statistical Determinations. Cell density was monitored by absorbance at 600 nm (OD_{600}) using a Biochrom Libra S22 spectrophotometer (Biochrom, Cambridge, UK). GC analysis [Agilent 7890A (Agilent, Santa Clara, CA) equipped with a flame ionization detector] for the quantification of α,ω-DCAs[7] and HPLC analysis [Shimadzu HPLC station (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (Shimadzu) and a SIL-20A autosampler (Shimadzu)] for quantification of sugars and xylitol[26] in the cell-free supernatant from the culture media of recombinant *E. coli* strains were performed as described previously. Product identification was performed on GC (Agilent 7890B) paired with a mass selective detector (MSD, Agilent 5977B). The expected reaction products were identified by their characteristic mass fragmentation pattern and compared to authentic standards. Substrate conversion efficiency was calculated on a molar basis. NADPH and NADP$^+$ were extracted and measured using a NADPH/NADP$^+$ quantitation kit (Sigma-Aldrich) following the manufacturer's instructions. All experimental data were subjected to one-way analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA) followed by Tukey's test using SPSS (Version 11) software (SPSS Inc., Chicago, IL) to determine levels of significance. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of Redox Balance on TDDA Production. Strains GXER (expressing none of the heterogeneous genes), GXER-M (expressing the CYP450 monooxygenase system and AlkL), and GXERω (expressing the CYP450 monooxygenase system, AlkL, ADH, and ALD) were evaluated for their ability of producing TDDA from C14-FA (Figure 1). GXER alone did not produce TDDA from C14-FA. It was found that there was no significant difference in TDDA titer between GXER-M (0.35 g/L) and GXERω (0.40 g/L) (Figure 1). The TDDA production of GXER-M from C14-FA indicates that the conversion of HFA to DCA not only resulted from the overoxidation activity of the heterologous expressed CYP450 system, but rather by the endogenous enzymatic machinery in the cell.[10,33] The possible reason for not increasing TDDA titer much in both the strains might be due to the redox imbalance. Since *xylAB* was deleted in GX strain, it is confirmed that there was no effect of xylose metabolism on DCA production.

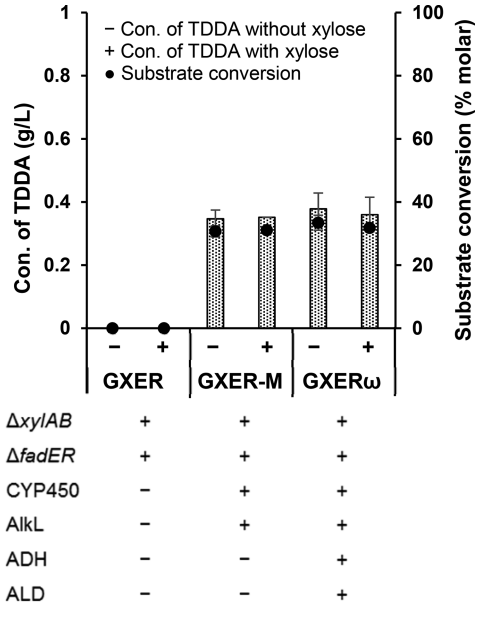

Figure 1. TDDA production by control strains. GXER, GXER-M, and GXERω strains were compared for their production of TDDA from C14- FA (1 g/L). No TDDA was formed in the wild type strain (GXER). The biotransformation was carried either with or without xylose. Gene deletions and enzymes overexpressed in each strain are indicated in the table. Data represent the mean of three different experiments, and error bars represent standard deviation.

A wild-type NADPH-dependent XR[30] (called XR$_{NADPH}$) from *Candida boidinii* was cloned, and a mutant NADH-dependent XR (called XR$_{NADH}$) with 10^4-fold higher specificity for NADH[27] was constructed from wild-type XR$_{NADPH}$ and expressed in the GXERω strain (Figure 2). These XR enzymes were expressed to facilitate NAD(P)H/NAD(P)$^+$ redox balance through the conversion of xylose and NAD(P)H to xylitol and NAD(P)$^+$. To evaluate the effect of NAD(P)H-dependent XR on TDDA production, four different medium conditions were tested, which varied with respect to the available cosubstrates, 0.4% each of Glu or Xyl: Glu$^+$Xyl$^+$ (group I), Glu$^-$Xyl$^+$ (group II), Glu$^+$Xyl$^-$ (group III), and Glu$^-$Xyl$^-$ (group IV). Other parameters, including the concentrations of the biocatalyst (50 g_{cww}/L) and substrate (1 g/L C14-FA), remained constant. GXERω strains expressing XR showed higher titers than those of GXERω (Figure 2). It is also noteworthy that the presence of glucose caused a significant increase in the conversion rate of FA to TDDA in all the strains (GXERω-XR$_{NADH}$, GXERω-XR$_{NADPH}$, and GXERω) tested regardless of the presence of XR and xylose. Glucose metabolism provides energy as well as precursors of cell components. Walton and Stewart reported that glucose can supply reducing equivalents to whole *E. coli* cells expressing cyclohexanone monooxygenase under non-growing conditions.[19]

The maximum TDDA production was obtained in GXERω expressing XR$_{NADPH}$ in the presence of glucose and xylose with 89% substrate conversion efficiency (Figure 2), compared with 36% for the DCA-producing strain REω.[7] It was expected that GXERω-XR$_{NADH}$ could produce higher concentration of TDDA production than GXERω-XR$_{NADPH}$ because of the redox cofactor specificity of the two dehydrogenases (ADH and ALD) producing NADH, not NADPH. A possible reason for the significantly less production of TDDA by GXERω-XR$_{NADH}$ compared with GXERω-XR$_{NADPH}$ could be due to the less activity of the mutated XR$_{NADH}$ compared with the wild-type XR$_{NADPH}$. Additionally, the consumption of NADH under high aeration conditions would be greater than under low aeration conditions, which could cause the NADH/NADPH ratio to decrease.[30] The same trend was also reported by Godoy and co-workers.[34] In the present study, the effect of aeration on DCA production was also tested using three different conditions: high aeration (250 rpm, 10 mL medium in 250 mL flask), medium aeration (250 rpm, 10 mL medium in 100 mL flask), and low aeration (125 rpm, 10 mL medium in 25 mL cylindrical bottle). The rotational agitation (rpm) and the relationship between the volume of culture medium and the volume of the flask are related to the concentration of dissolved oxygen, which plays an important role in fermentation.[34,35] In the present study, GXERω-XR$_{NADPH}$ produced approximately 1, 0.26, and 0 g/L TDDA under high-, medium-, and low-aeration conditions, respectively, while GXERω-XR$_{NADH}$ produced 0.44 g/L TDDA under high-aeration conditions

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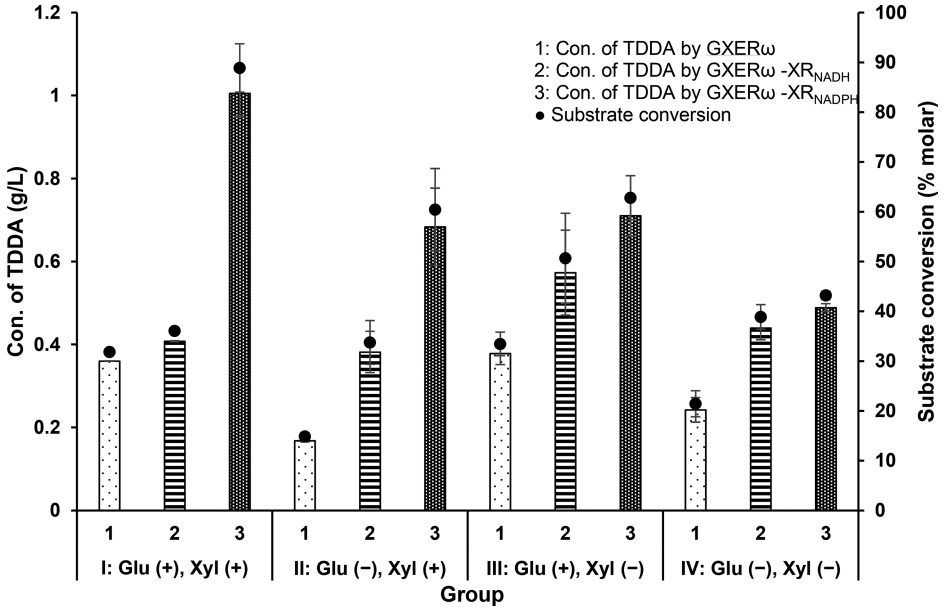
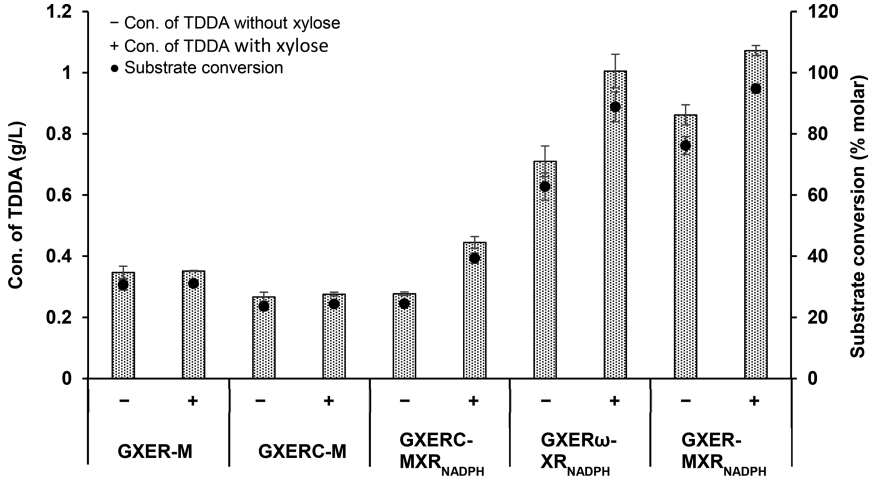


Figure 2. Effect of sugars and XR on TDDA production. Three strains, GXERω, GXERω-XR$_{NADH}$, and GXERω-XR$_{NADPH}$, were cultured in four different medium conditions (Group I to IV) varied in terms of available cosubstrates. Other parameters, including the concentrations of biocatalyst (50 g_{cww}/L) and substrate (1 g/L C14-FA), remained constant. Data represent the mean of three different experiments, and error bars represent standard deviation.



Δ*xylAB*	+	+	+	+	+
Δ*fadER*	+	+	+	+	+
Δ*puuC*	−	+	+	−	−
CYP450	+	+	+	+	+
AlkL	+	+	+	+	+
ADH	−	−	−	+	−
ALD	−	−	−	+	−
XR$_{NADPH}$	−	−	+	+	+

Figure 3. Increasing TDDA titer by the involvement of XR. TDDA production was evaluated either with or without xylose in different strains to determine the role of XR on the final titer. Gene deletions and enzymes overexpressed in each strain are indicated in the table. Data represent the mean of three different experiments, and error bars represent standard deviation.

and 0 g/L TDDA under medium- and low-aeration conditions. NADPH/NADP$^+$ and NAD$^+$/NADH ratios have been observed to be high under high-aeration conditions and low in nonaerated cultures.[34] Indeed, *in vivo* NADPH regeneration is an aerobic process.[22] Godoy and co-workers observed that the NADPH/NADP$^+$ ratio varies over a much wider range than the NADH/NAD$^+$ ratio with varying oxygen availability; variations in the NADH/NAD$^+$ ratio are found to be more discrete.[34] The NADP(H) pool is mostly reduced in highly aerated cultures and mostly oxidized in nonaerated cultures.[34] The TDDA concentration produced by GXERω-XR$_{NADPH}$ in a reaction condition only supplemented with xylose, not glucose, was not lower than that produced by the GXERω-XR$_{NADH}$ or control strain, indicating that competition between the

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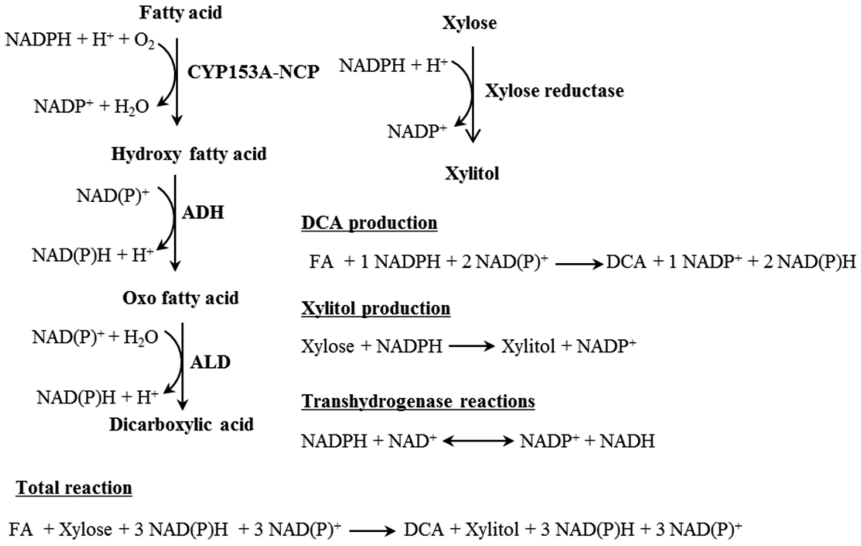

Figure 4. Metabolic pathways involved in the coproduction of DCA and xylitol by our system. XR is involving in the cofactor regeneration and balancing redox.

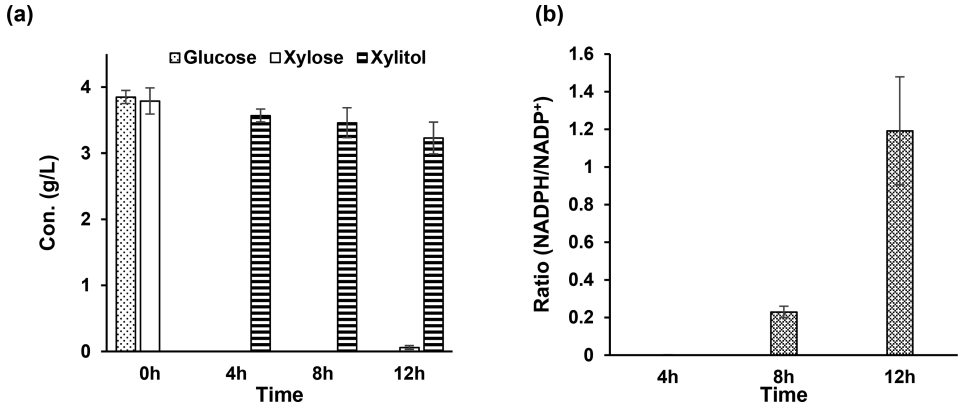

Figure 5. Coproduction of xylitol. (a) Xylitol concentration and (b) NADPH/NADP$^+$ ratio in GXER-MXR$_{NADPH}$ were quantified using C14-FA as a substrate. Data represent the mean of three different experiments, and error bars represent standard deviation.

monooxygenase and XR for use of NADPH as a cofactor was not significant because of sufficient NADPH/NADP$^+$ availability. Taking all these facts into account, we hypothesized that XR was involved in balancing redox in the strains.

Role of XR in TDDA Production. Figure 2 shows that GXERω strains with XR expression produced higher titers than strains without XR expression irrespective of the presence of its substrate xylose (Figure 2). Moreover, it was found that GXER-MXR$_{NADPH}$ produced 2.5-fold increased TDDA than GXER-M even in the absence of xylose (Figure 3). However, the mechanism by which the XR improves the TDDA titer is unclear. There may be two possible mechanisms: (i) XR has the activity toward any of the cellular metabolites rather than xylose resulting in redox cofactor regeneration; (ii) XR has enzyme activity to oxidize intermediates of the ω-oxidation pathway to yield DCA. The substrate specificity of XR from *C. boidinii* as an oxidase and a reductase has been studied using various aldehydes (xylose, valeraldehyde, propionaldehyde, methylglyoxal, glutaraldehyde, benzaldehyde, L-arabinose, fucose, ribose, and galactose) and alcohols (xylitol, L-arabitol, butanol, and propanol).[28,36] Further study should be necessary to elucidate these possibilities.

Bowen and co-workers[33] reported the existence of at-least five-ALDs (*puuC*, *gabD*, *feaB*, *patD*, and *astD*) showing ALD activity toward OFA in *E. coli*. The endogenous ADH and ALD use either NAD$^+$ or NADP$^+$ to oxidize HFA/OFA to yield DCA. The major ALD showing the activity on C12- and C14-OFA, *puuC*,[33] was deleted from GXER and compared its TDDA production with its counterparts. The *puuC* deletion in GXER-M expressing the CYP450 monooxygenase system and AlkL exhibited an at-least 1.3-fold decrease in TDDA production (Figure 3), indicating that *E. coli* has strong endogenous ALD activity. However, the XR expression in the *puuC* deleted strain did not increase TDDA production in the absence of xylose, indicating that the XR may not be involved in the ω-oxidation. However, a 1.6- fold increase in TDDA titer by GXERC-MXR$_{NADPH}$ was observed in the presence of xylose. The reason for this observation is the action of XR$_{NADPH}$ through cofactor regeneration (NADP$^+$) by reducing xylose to xylitol and providing cofactors for the catalytic action of NADP$^+$ dependent-ADH/ALD. Moreover, the cofactor specificity (NAD(P)$^+$) of ADH/ALD might be satisfied by several endogenous pyridine nucleotide transhydrogenases (TH) through interconversion of NAD(P)H and NAD(P)$^+$[34,37] (Figure 4). Transhydrogenases such as PntAB/UdhA, which are involved in maintaining redox equivalents, regulate the balance of the [NADPH + NAD$^+$]/[NADP$^+$ + NADH] ratio,

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Table 2. Effect of Increased Concentrations of Co-substrates, FAs, or Biocatalyst on TDDA Titer[a]

initial concentration	addition in 4 h of biotransformation	addition in 8 h of biotransformation	maximum TDDA con. (g/L)[b]
0.4% Glu + 0.4% Xyl + 1.5 g/L FA[c]			1.01 ± 0.05 (58.98%)
0.4% Glu + 0.4% Xyl + 2 g/L FA			1.01 ± 0.03 (44.60%)
1% Glu + 1% Xyl + 2 g/L FA			
2% Glu + 2% Xyl + 2 g/L FA			
1% Glu + 0% Xyl + 2 g/L FA	1% Xyl		
1% Glu + 0% Xyl + 2 g/L FA		1% Xyl	
1% Glu + 0% Xyl + 2 g/L FA	0.4% Xyl	0.4% Xyl	
1% Glu + 0% Xyl + 2 g/L FA	0.4% Xyl	0.4% Glu + 0.4% Xyl	
0.4% Glu + 0.4% Xyl + 1 g/L FA	0.2% Glu	0.2% Glu + 0.2% Xyl + 1g/L FA	0.648 ± 0.02 (57.27%)
0.4% Glu + 0% Xyl + 1 g/L FA	0.2% Glu + 0.4% Xyl	0.2% Glu + 0.2% Xyl + 1g/L FA	0.545 ± 0.02 (48.08%)
0.4% Gly[d] + 0.4% Xyl + 1 g/L FA			0.806 ± 0.01 (71.15%)
0.4% Glu + 0.4% Xyl + 2 g/L FA			1.00 ± 0.03 (44.53%)[e]

[a]All biotransformations were carried out in 10 mL of KPi buffer medium with GXER-MXR$_{NADPH}$ strain in a shaking incubator (30 °C and 250 rpm). [b]Concentration of TDDA calculated in 12 h of biotransformation. Substrate conversion efficiency (%) on molar basis is given in the parentheses. [c]FA:C14-FA. [d]0.4% Glycerol. [e]Cell concentration was 100$_{gwcw}$/L; TDDA concentration was calculated in 8 h of biotransformation.

which represents the overall balance of cofactors.[37] Therefore, strain GXER-MXR$_{NADPH}$ was selected for further study.

The use of a cofactor-balanced system in GXER-MXR$_{NADPH}$ resulted in a 3.4-fold increase in TDDA production with 95% substrate conversion efficiency from 1 g/L of C14-FA, compared with 32% for GXERω (Figure 1 and 3). In the case of DDDA, GXER-MXR$_{NADPH}$ exhibited a 3.6-fold increase in DDDA production with 67% substrate conversion efficiency, compared with 19% for GXERω. GXER-MXR$_{NADPH}$ produced the maximum amount of DCA (approximately 6% higher substrate conversion efficiency) than GXERω-XR$_{NADPH}$ (Figure 3). The presence of enzymes with affinity for different cofactors within a single pathway may affect the overall performance of biocatalytic processes under growth conditions that are not suitable for NAD(P)H regeneration.[20]

Coproduction of Xylitol and Advantages of Our System. In our system, a simple sugar, xylose, was used for redox balance, resulting in production of a valuable coproduct, xylitol ($C_5H_{12}O_5$), in addition to DCAs (Figure 4). Xylitol is a five-carbon sugar alcohol with global markets, and is mainly used as an alternative sweetener with beneficial health effects.[38] As the biotransformation system showed a higher titer and substrate conversion efficiency when C14-FA was used as the substrate, quantification of glucose, xylose, xylitol, and NADPH/NADP$^+$ was performed using GXER-MXR$_{NADPH}$, and C14-FA as the substrate. GXER-MXR$_{NADPH}$ produced a maximum of 3.57 g/L xylitol after a 4 h biotransformation (Figure 5a). Figure 5b shows the NADPH/NADP$^+$ ratio during biotransformation of C14-FA by GXER-MXR$_{NADPH}$. After a 4 h biotransformation, approximately 95% xylose was converted to xylitol, with simultaneous conversion of 50% FA to DCA. Therefore, another advantage of the system presented here is the coproduction of xylitol, an industrially important chemical that is considered nontoxic to the cell and also secreted outside the cell.

For cofactor (NADPH) regeneration, several dehydrogenases, such as glucose dehydrogenase,[39,40] isocitrate dehydrogenase,[41] short-chain ketone dehydrogenase,[19] and formate dehydrogenase,[42] have been utilized in whole-cell *E. coli*-mediated biocatalytic processes in both monophasic[39] and biphasic systems,[40] suggesting that both enzymes involving in the main biosynthetic pathway and cofactor regeneration can be used in combination. As suggested elsewhere,[7,11,18,25] in the present study, a cofactor-regenerating system was used to increase the overall performance of the whole-cell biotransformation, without compromising the product titer, resulting in enhanced production of DCAs from renewable FA substrates. The use of a second enzyme for cofactor regeneration is more prevalent than the co-option of cellular metabolism[20,22,39] because cellular metabolism has a limited capacity for cofactor regeneration. It has been reported that the regeneration of NADPH remains a major challenge for the efficient application of CYP450s.[18−20,25] *E. coli* cells would be able to support even more efficient NADPH-dependent bioconversions if a more suitable enzyme−substrate pair could be identified.[19] More recently, Beyer and co-workers used phosphite dehydrogenase to regenerate NADPH via oxidation of cheap phosphite to phosphate in a CYP450$_{BM3}$-mediated biocatalytic process.[20] In the case of NADH regeneration, a NAD$^+$-dependent formate dehydrogenase from *C. boidinii* has been expressed in *E. coli* to maximize production of ω-hydroxyhexadecanoic acid by a NADH-dependent CYP450.[25]

It was found that addition of cosubstrates (0.4% each of Glu and Xyl) favored the biotransformation (Figure 2). Several reports show the addition of cosubstrates such as glucose and/or glycerol at multiple time points during a biotransformation by the resting cells to foster the redox cofactor regeneration/availability.[10,16,18] When compared to that of other reports,[10,18] in the present study, cosubstrates (glucose and xylose) were added only at the start of the biotransformation and moreover achieved around 95% substrate conversion with a use of a four-fold less concentration of cosubstrates in 12 h of biotransformation. Scheps and co-workers produced 1.2 g/L of C12-HFA from 10 g/L of C12-FA (around 11% substrate conversion) with a supplementation of a 30 h whole-cell biotransformation reaction with a total of 3.2% cosubstrates (glucose and glycerol).[10] Therefore, the present study shows the efficiency and applicability of introducing cofactor regeneration systems to address the problem of redox imbalance in CYP450-catalyzed whole-cell biotransformations.

In addition, genetic engineering strategies such as blocking cofactor-consuming pathways or modifying central metabolic pathways sometimes result in growth retardation or even redox imbalance, thereby causing reductions in target enzyme expression and lower product titers.[43,44] Moreover, water-forming NADH oxidase (NOX) has been applied for cofactor regeneration in engineered whole-cell biocatalysts;[45] however, in this case, expression of NOX from *Lactococcus lactis* in a

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DCA-synthesizing strain (GXERω) did not yield an increase in DCA production (data not shown). NOX activity was found to be strain-dependent and growth condition-dependent, leading to significant differences in product titers among different whole-cell biocatalysts.[45] It is noteworthy that the XR system presented here can produce the value-added chemical, xylitol, using accumulated NADPH rather than just oxidizing NAD(P)H like NOX.

Effect of Carbon and Energy Source, Substrate, and Biocatalyst Concentrations on TDDA Production. To investigate the influence of environmental and nutritional factors on TDDA production, the effect of concentrations of initial FA substrate (0.5, 1, 1.5, and 2 g/L C14-FA), sugar (0.4%, 1%, or 2% of Glu and/or Xyl, or 0.4% glycerol), and biocatalyst (5−100 g_{wcw}/L) on the final titer of TDDA was evaluated. Table 2 shows that increases in sugar concentration during the biotransformation did not increase the product concentration. The glucose and xylitol (0.4% each) that were initially added to the reaction mixture were used up within 4 h of biotransformation (Figure 5a). The use of higher sugar concentrations did not increase final TDDA concentrations greater than 1 g/L from 1.5 g/L, or 2 g/L C14-FA. Use of glycerol instead of glucose resulted in at least a 25% decrease in TDDA production. Bennett and co-workers showed that NAD(P)H concentrations in aerobic, exponentially growing *E. coli* cells differ significantly depending on whether glucose or glycerol is used as the carbon and energy source.[46] The maximum titer (1 g/L of TDDA) was achieved within 8 h with 100 g_{wcw}/L of initial biocatalyst concentration, when compared within 12 h with 50 g_{wcw}/L of initial biocatalyst concentration (Table 2). However, the concentration of TDDA was found to be dependent on the concentration of biocatalyst up to 50 g_{wcw}/L, beyond which there was no increase in product concentration (Figure 6a).

Figure 6b shows the substrate conversion and maximal production rates of DDDA and TDDA from their respective FAs at different time points. The maximal production rate (R_{max}) was found to be higher at the earlier stage of the biotransformation reaction. In the case of DDDA, the low titer and substrate conversion efficiency could be due to the lower substrate conversion efficiency for C12-FA (60−65%) than C14-FA (80−85%)[7,47] by CYP153A monooxygenase, or substrate inhibition (K_{si} 2 mM).[18] Moreover, these differences may have been due to the longer chain length of the FA. FAs with shorter chain lengths are more detrimental to the cell membrane of *E. coli* than FAs with long chain lengths (>C12).[48] It is noteworthy that almost 100% substrate conversion efficiency was achieved when low substrate concentrations (0.25 g/L C12-FA or 0.5 g/L C14-FA) were used (Figure 6c).

The FA uptake was facilitated by the following strategies based on previous reports: use of cosolvent (DMSO) and surfactants (Tween 80), which are considered to enhance substrate solubility or membrane permeability;[13,49] expression of the outer membrane protein AlkL from *Pseudomonas putida* and deletion of fadR.[13] It was found that the AlkL can improve the fatty acid uptake, resulting in high productivity.[10,13] An outer-membrane-bound fatty acid transport protein FadL and inner-membrane-associated protein FadD are responsible for transport of exogenous long-chain fatty acids into the cell by a specific transport process. These two genes were repressed by *fadR*.[50] Cho and co-workers[51] found that the expression levels of *fadL* and *fadD* were elevated 4- and 3-fold, respectively, in

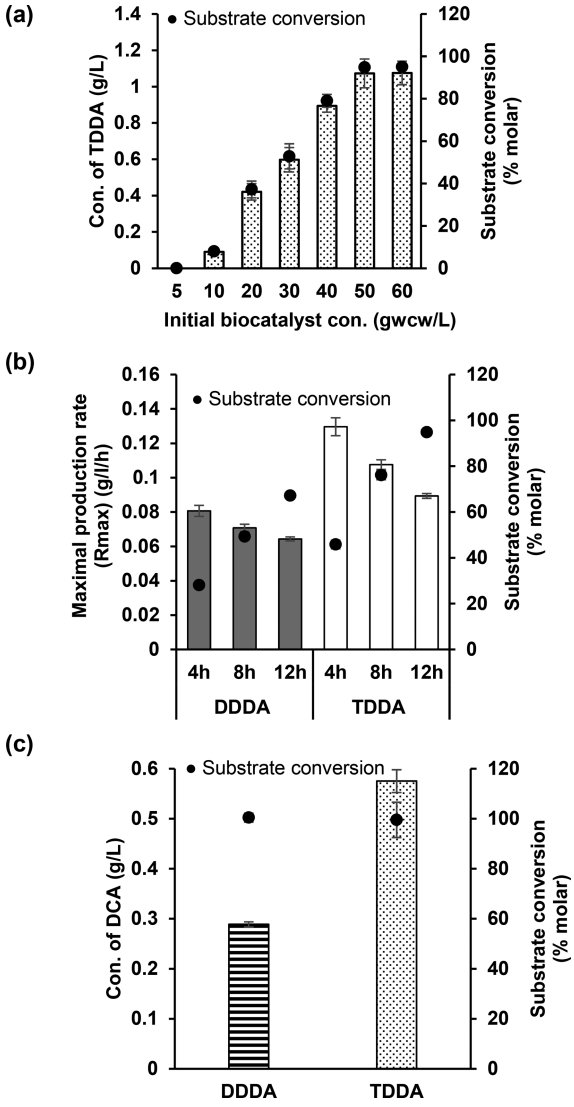

Figure 6. Influence of the concentrations of biocatalyst and substrate on the DCA production by GXER-MXR_{NADPH}. (a) The influence of the concentration of biocatalyst on the TDDA concentration was studied with different concentrations of initial biocatalyst (g_{wcw}/L) using C14-FA as a substrate (1 g/L). (b) Production of DDDA and TDDA by GXER-MXR_{NADPH} from 1 g/L C12-FA or C14-FA, respectively, was evaluated. Samples were taken at different time points for GC analysis. R_{max} (g/L/h) was found to be higher at the early stages of the biotransformation. (c) Almost 100% substrate conversion to DCAs was obtained when low substrate concentrations (0.25 g/L C12-FA or 0.5 g/L C14-FA) were used. Data represent the mean of three different experiments, and error bars represent standard deviation.

the Δ*fadR* mutant. In our previous study,[7] it was reported that the double knockout strain (*fadR* and *fadE*) produced 142% more C12 DCA and 204% more C14 DCA than single knockout strain (*fadE*) and suggested that *fadR* deletion facilitates the transport of fatty acids. The degradation of imported fatty acids is avoided by deleting the critical conversion step carried out by acyl-CoA dehydrogenase (*fadE*), thus preventing the β-oxidation pathway.

A reduced substrate conversion (<50%) was obtained at higher concentrations of C14-FA (2 g/L). A possible reason for the observation may be substrate toxicity to the cells and enzymes.[13,18,52] Kitazume and co-workers reported that

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substrate inhibition occurred at higher concentrations for longer FA substrates (≥C13) with CYP450 of *Fusarium oxysporum*, suggesting that inhibition depended on the binding of a second fatty acid to form an abortive enzyme−substrate complex.[52] It has also been reported that the oxyfunctionalized intermediates or products may contribute to reduced viability and biocatalytic activity even at low concentrations.[13] As suggested elsewhere[10,13,16,18] to avoid substrate and product inhibition, the extractive biotransformation could be adapted to a biphasic process to serve as a potent and sustainable platform for the production of high concentrations of α,ω-DCAs.

To conclude, in the present study, DCA production was enhanced by employing an enzymatic XR-dependent NADP-(H)-regenerating system in engineered *E. coli* strains. The cofactor-complemented system results in the production of TDDA (1 g/L) with 95% substrate conversion efficiency, with a 3.4-fold increase in TDDA concentration compared with the noncomplemented system. In addition, the strain GXER-MXR_{NADPH} produced a maximum of 3.57 g/L xylitol from 4 g/L xylose. These results suggest that the system presented here has potential applications for the large-scale production of α,ω-DCAs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00376.

Primers used in this study (PDF)

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Notes

The authors declare no competing financial interest.

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