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Furan carboxylic acids production with high productivity by cofactor-engineered whole-cell biocatalysts

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Abstract: Furan carboxylic acids are useful chemicals in various industries. In this work, biocatalytic production of furan carboxylic acids was reported with high productivities by cofactor-engineered *Escherichia coli* cells. NADH oxidase (NOX) was introduced into *E. coli* harboring aldehyde dehydrogenases (ALDHs) to promote intracellular NAD^+ regeneration, thus significantly enhancing ALDH-catalyzed oxidation. These engineered biocatalysts were capable of efficient aerobic oxidation of a variety of aromatic aldehydes. More importantly, they exhibited high substrate tolerance toward toxic furans. *E. coli* co-expressing vanillin dehydrogenase and NOX (*E. coli*_CtVDH1_NOX) enabled efficient oxidation of 250 mM of 5-hydroxymethylfurfural (HMF) to 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), providing a productivity of 3.7 g/L h. With *E. coli*_CtVDH2_NOX as catalyst, up to 240 mM of furfural and 5-methoxymethylfurfural (MMF) could be smoothly oxidized. 2-Furoic acid (FCA, 227 mM) and 5-methoxymethyl-2-furancarboxylic acid (MMFCA, 287 mM) were produced in fed-batch synthesis, providing the productivities of 2.0 and 5.6 g/L h, respectively.

Introduction

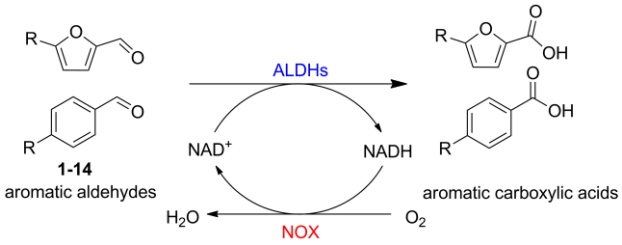
In the last decades, the production of chemicals and fuels from biomass has attracted growing interest, due to increased concerns on environmental issues (e.g., global warming).[1] Biobased furans such as 5-hydroxymethylfurfural (HMF) and furfural are recognized as top-value biobased platform chemicals,[2] which are synthesized via carbohydrate dehydration. 5-Methoxymethylfurfural （MMF), a structural analog of HMF, is produced in the presence of methanol via carbohydrate dehydration.[3] These furans can be upgraded to value-added chemicals via typical chemical transformations such as hydrogenation, oxidation, esterification, and amination.[4] For example, 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), one of oxidation derivatives of HMF, is a versatile building block for the synthesis of polyesters[5] and terephthalic acid (TPA)[6] as well as an interleukin inhibitor.[7] It also shows antitumor activity.[8] 2-Furoic acid (2-furancarboxylic acid, FCA) derived from furfural has found wide applications in pharmaceutical, agrochemical, flavor and fragrance industries.[9] Also, 5-methoxymethyl-2-furancarboxylic acid (MMFCA) is a starting material for the synthesis of renewable TPA[6] and 2,5-furandicarboxylic acid (FDCA)[10], both of which were valuable monomers in the fields of polyesters, polyamides and polyurethanes.[11]

In terms of environmental friendliness, mild degree of reaction conditions, and selectivity, biocatalysis appears to be advantageous over chemical counterpart.[12] Especially, biotransformations that usually proceed under mild conditions may be preferred for the valorization of inherently unstable biobased furans.[13] Indeed, biocatalytic upgrading of biobased furans has been of great interest.[14] Many whole cells[15] as well as isolated enzymes[16] were exploited for selective oxidation of these furans to furan carboxylic acids. Although some proof-of-concept studies were previously reported,[16b, 17] biotransformation of biobased furans remains a fundamental challenge, because these furans displayed significant toxicity and inhibition toward biocatalysts.[18] The unsatisfactory substrate tolerance and low productivity of biocatalysts reported previously may be the major barriers for their large-scale

applications. Previously, our group presented a proof-of-concept study on biocatalytic selective oxidation of HMF to HMFCA using *Comamonas testosteroni* SC1588 cells.[15a] Strikingly, Zhang and co-workers recently reported a high HMF-tolerant strain (up to 300 mM) *Deinococcus wulumuqiensis* R12 for the oxidation of HMF to HMFCA; the product of up to 511 mM was synthesized within 20 h in a fed-batch process, leading to a volumetric productivity of 3.6 g/L h.[19] Pyo and co-workers described the fed-batch synthesis of HMFCA using *Gluconobacter oxydans* DSM 50049 cells, with a volumetric productivity of approximately 2 g/L h.[15e] Zhou et al. found that *G. oxydans* ATCC 621H enabled efficient synthesis of FCA from furfural that is more toxic toward whole-cell biocatalysts than HMF, with a volumetric productivity of 1.6 g/L h.[20] Recently, our group reported whole-cell catalytic synthesis of HMFCA and MMFCA using *Escherichia coli* overexpressing aldehyde dehydrogenases (*E. coli*_ALDHs), with the volumetric productivities of 2 and 1.2 g/L h, respectively.[15c, 18]

ALDHs have recently proven to be useful catalysts in synthetic chemistry by us and others,[15c, 15d, 18, 21] which enable the oxidation of aldehydes to carboxylic acids with oxidized nicotinamide cofactors ($NAD(P)^+$). Aldehyde bind to the ALDH-$NAD(P)^+$ complex to form the thiohemiacetal, followed by oxidation to an acyl intermediate; finally, the deacylation occurs, thus releasing carboxylic acid.[22] We found that, in addition to furan carboxylic acids, minor furan alcohols (10-20%) were formed as byproducts at the initial stage in the oxidation of furan aldehydes by *E. coli*_ALDHs, and then these byproducts were slowly re-oxidized into acids, thus markedly reducing the transformation efficiencies.[15c, 21a] The reason for the formation of minor byproducts might be that NADH produced in ALDH-catalyzed oxidation could not be efficiently oxidized to NAD^+ *in vivo*, and thus it was utilized by unspecific dehydrogenases present in host cells to reduce aldehydes. Therefore, we envisioned that the byproduct formation would be significantly alleviated by a cofactor engineering strategy, which is capable of effectively promoting the conversion of intracellular NADH to NAD^+.[23] NADH oxidase (NOX) is able to oxidize NADH to NAD^+ with O_2, producing H_2O or H_2O_2 as byproduct.[24] In this work, therefore, we engineered

intracellular redox environment of *E. coli*_ALDHs through the introduction NOX from *Lactobacillus brevis* into these biocatalysts (Scheme 1), thus greatly intensifying catalytic oxidation of aldehydes. The substrate scope of these cofactor-engineered biocatalysts and their tolerance toward furans were examined. Additionally, the fed-batch synthesis of three valuable furan carboxylic acids including HMFCA, FCA and MMFCA was performed to achieve high productivities.



Scheme 1. Oxidation of aldehydes to carboxylic acids by cofactor-engineered whole-cell biocatalysts

Materials and Methods

Chemical and biological materials

Protein marker, restriction endonucleases, T4 DNA ligase, and MMFCA (99%) were purchased from Thermo Fisher Scientific GmbH (Schwerte, Germany). LA *Taq* Hot Start polymerase was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The reagent kits for constructing recombinant plasmids were purchased from Generay Biotech Co., Ltd (Shanghai, China). Isopropyl β-D-1-thiogalactopyranoside (IPTG), DNA marker, and ampicillin were purchased from Sangon Biotech (Shanghai, China). FDCA (97%), and 5-formyl-2-furancarboxylic acid (FFCA, 98%) were purchased from J&K Scientific Ltd. (Guangzhou, China). HMFCA (98%), and MMF (97%) were bought from Adamas Reagent Ltd. (Shanghai, China). Furfural (99%), and 2,5-bis(hydroxymethyl)furan (BHMF, 98%) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). 2,5-Diformylfuran (DFF, 98%), 5-methylfurfural (97%), furfuryl alcohol (98%), and FCA (98%) were purchased from TCI (Japan). HMF (98%) was purchased from Aladdin (Shanghai, China). 5-Methyl-2-furoic acid (97%) was obtained from Sigma-Aldrich (USA). The sources and purity of other chemicals are

available as Supporting Information. The strains, plasmids, and primers used in this study are presented in Tables S1 and S2, respectively. *C. testosteroni* SC1588 was maintained in our laboratory.[15a]

Construction and cultivation of recombinant biocatalysts

The ALDH genes *Ctcaldh*2 (GenBank Accession No. MN460794; encoding coniferyl aldehyde dehydrogenase 2, CtCALDH2), *Ctvdh*1 (GenBank Accession No. MN460795; encoding vanillin dehydrogenase 1, CtVDH1), *Ctvdh*2 (GenBank Accession No. MN4607946; encoding vanillin dehydrogenase 2, CtVDH2) and *Ctsapdh* (GenBank Accession No. MN460792; encoding 3-succinoylsemialdehyde-pyridine dehydrogenase, CtSAPDH) were amplified with genomic DNA of *C. testosteroni* SC1588 as template. Figure S1 shows the schematic diagrams of the construction of recombinant expression plasmids. Briefly, the PCR products were double-digested using the restriction enzymes BamH I and Hind III; then, the resulting products were inserted into the expression plasmid pETDuet-1, thus generating the recombinant plasmids pETDuet_*Ctaldh*. The *nox* gene (GenBank Accession No. ARW22543; encoding NOX) from *L. brevis* was amplified with pET28a_*nox* as template. The purified double-digestion fragment was inserted into the plasmids pETDuet-1 and pETDuet_*Ctaldh*, thus producing pETDuet_*nox* and pETDuet_*Ctaldh_nox*, respectively. Finally, the obtained plasmids were transformed into *E. coli* BL21 (DE3), affording recombinant whole-cell biocatalysts.

Recombinant *E. coli* strains were pre-cultivated overnight in 30 mL of Luria-Bertani (LB) medium containing 100 mg/L ampicillin at 37 °C and 180 r/min. Then, 100 mL LB medium containing 100 mg/L ampicillin was inoculated with 1 mL of the overnight culture, followed by cultivation at 37 °C and 180 r/min. When the optical density at 600 nm (OD_{600}) reached 0.6-0.8, IPTG (as inducer) was added to the medium at a final concentration of 0.1 mM, followed by induction at 20 °C and 160 r/min for 20 h. The cells were harvested by centrifugation (8000 r/min, 5 min, 4 °C) and washed twice with 0.85% NaCl solution.

Determination of intracellular NAD(H) concentrations

The intracellular concentrations of NAD^+ and NADH were determined by the reagent kit (Keming Bio-Tech Co., China), according to the manual.

Enzyme assay

For assaying ALDH activity, 11 g/L (cell dry weight, CDW) of cells were added into 4 mL of K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7) containing 100 mM HMF, and incubated at 30 °C and 150 r/min for 10 min. The formation of HMFCA was monitored by HPLC. One unit of enzyme activity (U) was defined as the amount of the cells (CDW) which produce 1 μmol HMFCA per minute under the above conditions.

The NOX activity was spectrophotometrically determined at 30 °C by monitoring the NADH oxidation at 340 nm.[25] The reaction mixture contains 0.2 mM NADH, an appropriate amount of crude enzyme (the supernatant of cell lysate), and K_2HPO_4-NaH_2PO_4 buffer (50 mM, pH 7). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADH per minute under the above conditions.

General procedure for biocatalytic oxidation of aldehydes

Typically, 4 mL of K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7) containing 100-280 mM of aldehydes and 4-22 g/L (CDW) of microbial cells was incubated at 30 °C and 150 r/min. Aliquots were withdrawn from the reaction mixtures at specified time intervals and diluted with the corresponding mobile phase prior to HPLC analysis. The conversion was defined as the ratio of the consumed substrate amount to the initial substrate amount (in mol). The yield was defined as the ratio of the formed product amount to the theoretical value based on the initial substrate amount (in mol). All the experiments were conducted at least in duplicate, and the values were expressed as the means ± standard deviations.

HPLC Analysis

The reaction mixtures were analyzed on a Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA) by using a reversed phase HPLC (Waters, USA). The detailed methods and HPLC spectra are available as Supporting Information.

Results and Discussion

Engineering whole-cell biocatalysts for HMF oxidation

With CtVDH1 as a model dehydrogenase, NOX was co-expressed with this enzyme in *E. coli* BL21 (DE3) for promoting NAD^+ recycling (Figure S1). *E. coli* without NOX was used as the control. Based on SDS-PAGE analysis (Figure S2), soluble expression of these heterologous proteins was implemented in *E. coli*. These *E. coli* strains were exploited for the oxidation of HMF **1** (Figure 1a). As expected, the production of byproduct BHMF was significantly reduced with cofactor-engineered *E. coli* (*E. coli*_CtVDH1_NOX) as catalyst compared to that with *E. coli*_CtVDH1 (3% vs 12%, Figure 1b). More interestingly, the synthesis of the desired product HMFCA was considerably enhanced with the engineered cells; HMFCA was obtained with a 96% yield within 2 h (Figure 1b). It is in good agreement with previous reports.[23a, 26] To explain the obtained results, intracellular concentrations of NAD^+ and NADH were determined (Figure 1c). It was interestingly found that the concentration ratios of NAD^+ to NADH ($[NAD^+]$/[NADH]) reached 3.4 in *E. coli*_ CtVDH1_NOX, which is 2-fold higher than that (approximately 1.7) in the control. Higher intracellular $[NAD^+]$/[NADH] might not only significantly promote biocatalytic HMF oxidation, but also alleviate the HMF reduction, likely due to functional expression of NOX. To verify the assumption, the NOX activities of the cell supernatants as well as the ALDH activities of the recombinant cells were assayed (Figure 1d). As shown in Figure 1d, the cells without the incorporation of CtVDH1 displayed extremely low ALDH activities, whereas high ALDH activities (112-129 U/g) were observed with the strains expressing CtVDH1. More importantly, the cell supernatants of cofactor-engineered *E. coli* displayed high NOX activities (14-16 U/mg). In contract, almost no NOX activities were found in the strains without the introduction of NOX. Therefore, improved synthesis of HMFCA with *E. coli*_CtVDH1_NOX might be attributed to functional expression of NOX capable of efficiently recycling NAD^+ from NADH.

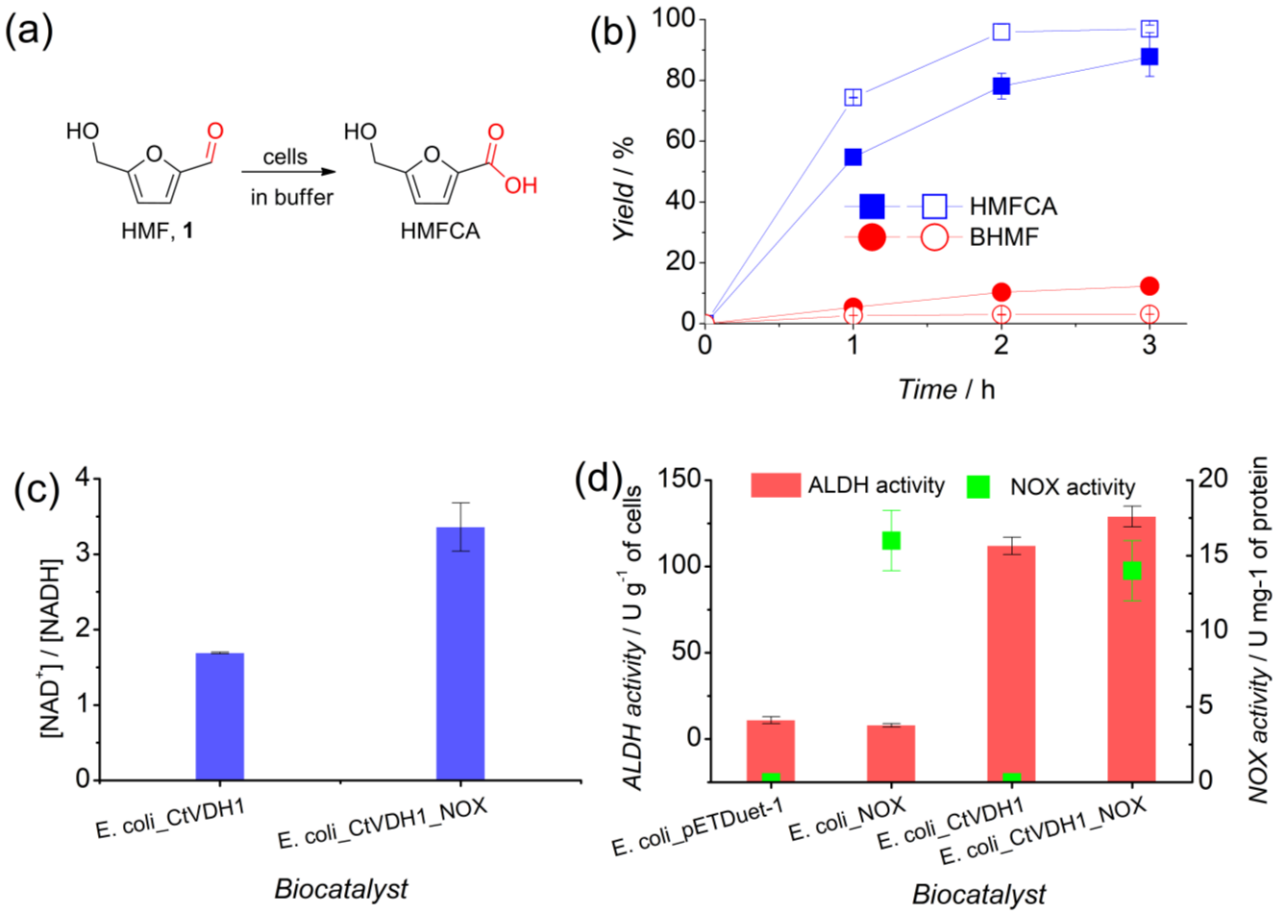


Figure 1. Biocatalytic oxidation of HMF **1** by recombinant cells (a), time courses of the oxidation reaction (b), intracellular concentration ratio of NAD^+ to NADH (c), and ALDH and NOX activities (d). Symbols in Figure 1b: *E. coli*_CtVDH1 (solid symbols), *E. coli*_CtVDH1_NOX (open symbols). Conditions for Figure 1b: 100 mM HMF, 11 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min; conditions for Figure 1c and d are available in experimental section.

Encouraged by the above results, other biocatalysts including *E. coli*_CtVDH2, *E. coli*_CtCALDH2, and *E. coli*_CtSAPDH were cofactor-engineered by introducing NOX (Figure S3). Also, functional expression of these proteins was readily achieved (Table S3). In the cases of *E. coli*_CtCALDH2 and *E. coli*_CtSAPDH, the introduction of NOX caused the reduced ALDH activities of the recombinant cells (Table S3, entries 5 and 8). For *E. coli*_CtCALDH2_NOX, a comparable ALDH activity was retained when its induction temperature was lowered to 16 °C (Table S3, entry 6). However, this strategy was ineffective for *E. coli*_CtSAPDH_NOX (Table S3, entry 9). Then, the catalytic performances of these engineered strains with good ALDH activities were

evaluated for the oxidation of HMF (Table 1). Both *E. coli*_CtVDH1_NOX and *E. coli*_CtVDH2_NOX provided the improved results in the HMF oxidation compared to their counterparts without cofactor engineering (Table S4). In addition, most engineered biocatalysts showed good catalytic activities and excellent substrate tolerance. Both *E. coli*_CtVDH1_NOX and *E. coli*_CtVDH2_NOX enabled efficient oxidation of up to 250 mM of HMF to HMFCA, with the yields of more than 92% (Table 1, entries 2 and 5). Compared to *E. coli*_CtVDH1 recently reported by us,[15c] *E. coli*_CtVDH1_NOX displayed higher substrate tolerance (200 vs 250 mM) and selectivity (92% vs 95%) in the batch process as well as a higher volumetric productivity (2.2 vs 3.7 g/L h).

Table 1. Whole-cell catalytic synthesis of HMFCA from HMF **1**

Entry	Catalyst	HMF conc. (mM)	Time (h)	HMF conv. (%)	HMFCA yield (%)
1	*E. coli*_CtVDH1_NOX	200	7	>99	97 ± 2
2	***E. coli*_CtVDH1_NOX**	**250**	**9**	**>99**	**95 ± 2**
3	*E. coli*_CtVDH2_NOX	100	2	>99	94 ± 2
4	*E. coli*_CtVDH2_NOX	200	9	>99	92 ± 1
5	***E. coli*_CtVDH2_NOX**	**250**	**12**	**>99**	**92 ± 1**
6	*E. coli*_CtCALDH2_NOX [a]	100	6	92 ± 1	89 ± 2
7	*E. coli*_CtSAPDH	100	2	>99	87 ± 2

Reaction conditions: 100-250 mM HMF **1**, 11 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min; tuning pH to 7 every 2 h using $NaHCO_3$.

[a]: Protein expression was induced at 16 ºC during cell cultivation.

Elucidation of substrate scope

To broaden their applications in synthetic chemistry, the substrate scope of these biocatalysts was studied, in which a group of aromatic aldehydes was tested (Figure 2). The four biocatalysts displayed good catalytic performances toward most of substrates. The yields of some target products were as high as >99%. Of the four biocatalysts tested, *E. coli*_CtVDH2_NOX proved to be the most versatile catalyst in the oxidation of these aromatic aldehydes; all target carboxylic acids were obtained with excellent yields (more than 91%). With DFF **2** as substrate, it could be sequentially oxidized to FFCA

3 and FDCA. As shown in Figure 2, DFF **2** was a good substrate of most of biocatalysts, except for *E. coli*_CALDH2_NOX, since its conversions reached 99% in 12 h (data not shown). In the oxidation of DFF **2**, the FDCA yields depended on the catalytic activities of biocatalysts toward FFCA **3**. *E. coli*_CtVDH2_NOX showed a good activity toward FFCA **3**, and thus a high FDCA yield (96%) was obtained in the oxidation of DFF **2** with this catalyst. In contract, *E. coli*_CtSAPDH with a modest catalytic activity toward FFCA **3** (leading to 63% yield of FDCA) provided a moderate FDCA yield (52%) in the oxidation of DFF **2**. Other furan derivatives including compounds **4-7** were well accepted as substrates by these catalysts; target furan carboxylic acids were afforded with good yields (84->99%).

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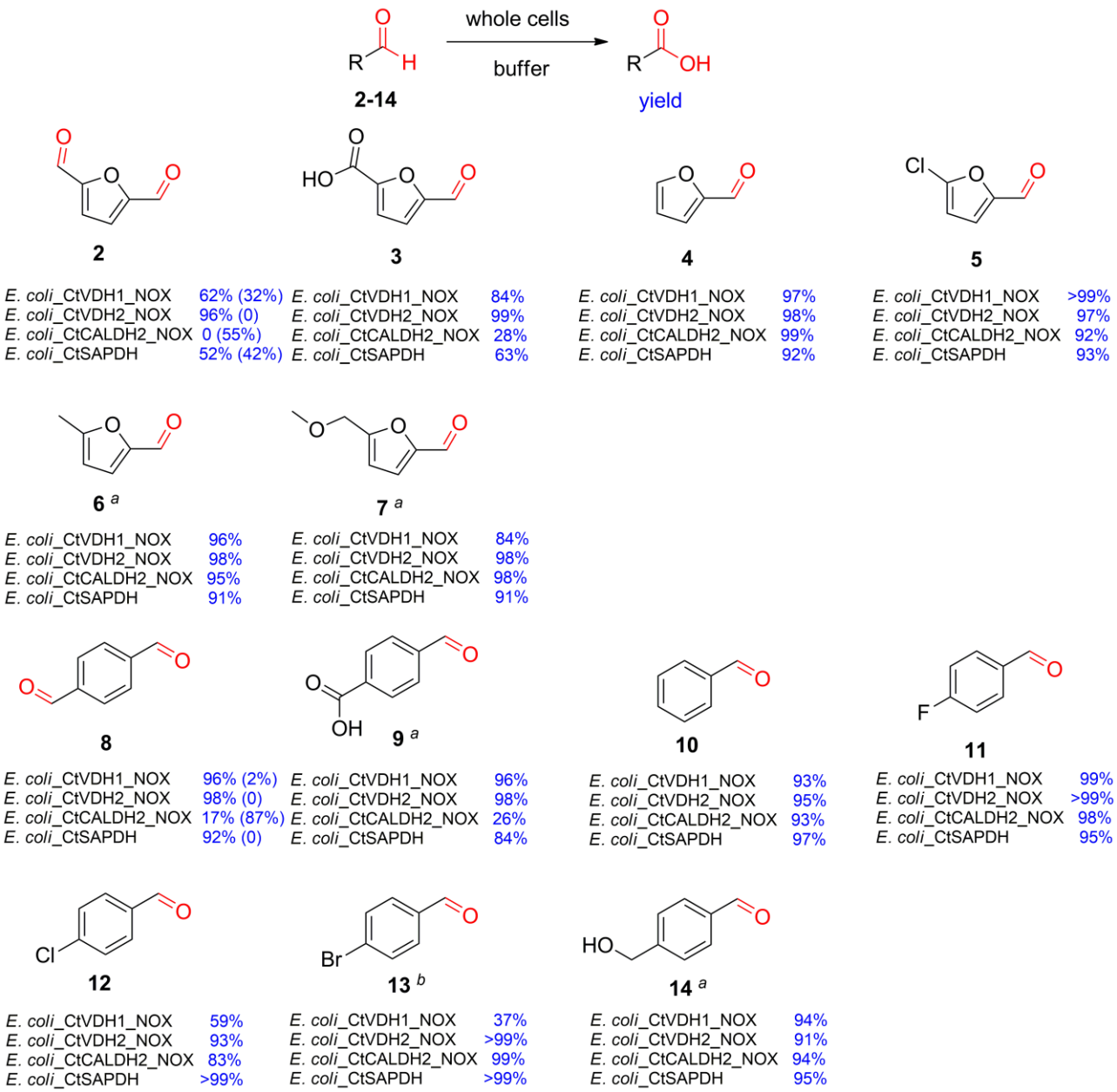

Figure 2. Substrate scope of these whole-cell biocatalysts. Reaction conditions: 20 mM substrate, 4 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min, 12 h. Yields of target acids were showed in blue font, and their standard deviations were less than 4%. In the oxidation of compounds **2** and **8**, the yields of the products **3** and **9** were given in parenthesis. Protein expression was induced at 16 ºC during the cultivation of *E. coli*_CtCALDH2_NOX.

[a]: 3 h.

[b]: 24 h.

Like DFF **2**, terephthalaldehyde **8** was oxidized to 4-formylbenzoic acid **9**, followed by oxidation to TPA. Compared to DFF **2**, its phenyl analog **8** seemed to be a preferred substrate for these biocatalysts. The substrate conversions reached >99% in all cases, and TPA was obtained in the yields of more than 92% with most of biocatalysts. The same effect was also observed in compounds **3** versus **9**, but not in compounds **5** versus **12**. As shown in Figure 2, the yields of benzoic acids were strongly dependent on the nature of the substituents on the phenyl ring. Benzaldehyde with a strong electron-withdrawing group (e.g., F atom, 4-fluorobenzaldehyde **11**) could be efficiently oxidized to target acid within 12 h, while a long reaction period (24 h) was required for complete oxidation of 4-bromobenzaldehyde **13** with a weak electron-withdrawing atom. For *E. coli*_CtVDH1_NOX, particularly, the effect of the substituent electronegativities on biocatalytic oxidation of benzaldehydes appeared to be more evident. The benzoic acid yields decreased from 99% to 37% with the reduced electronegativities of the substituents (F atom: 4.0; Cl: 3.0; Br: 2.8).[27] Also, 4-hydroxymethylbenzaldehyde **14**, a structural analog of HMF **1**, was a good substrate of these biocatalysts; 4-hydroxymethylbenzoic acid was furnished with good yields within 3 h (Figure 2).

Herein we provided a versatile catalytic toolbox for the oxidation of aromatic aldehydes to target carboxylic acids. For example, *E. coli*_CtVDH2_NOX exhibited high activities toward almost all substrates tested. Besides, *E. coli*_CtSAPDH is a preferred catalyst for the oxidation of compounds **12-14**. These whole-cell catalysts constructed in this work could tolerate a variety of functional groups such as the primary hydroxyl and halogen atoms, and they were capable of selectively oxidizing the formyl group(s) present in the substrates to the carboxyl group(s).

Batch and fed-batch synthesis of FCA

FCA is an important fine chemical, which has been widely used in the pharmaceutical, agrochemical, flavor and fragrance industries.[9] However, furfural **4** was not only strongly inhibitory but also greatly toxic toward biocatalysts.[18] Therefore,

the substrate tolerance of biocatalysts toward furfural **4** was evaluated, and the optimized synthesis of FCA was performed to achieve a good productivity (Table 2). Based on their substrate scope (Figure 2), three biocatalysts including *E. coli*_CtVDH1_NOX, *E. coli*_CtVDH2_NOX and *E. coli*_CtCALDH2_NOX were used for this purpose. As shown in Table 2, entry 1, *E. coli*_CtVDH1_NOX gave poor results in the oxidation of furfural **4**, despite its good catalytic performances in the oxidation of HMF **1** (Table 1). The reason may be that furfural is much more toxic and inhibitory toward the cells as well as toward dehydrogenases than HMF,[18, 28] especially at high concentrations, which may be closely related to its higher hydrophobicity. Interestingly, *E. coli*_CtVDH2_NOX proved to be an excellent catalyst for the oxidation of toxic furfural **4** (Table 2). FCA was obtained with the yields of 93% and 83% at the substrate concentrations of 100 and 150 mM, respectively (Table 2, entries 2 and 4). Significantly improved FCA synthesis was achieved by increasing cell concentrations. Furfural **4** of up to 240 mM was smoothly converted in 24 h in the presence of 22 g/L of cells, affording FCA with a 90% yield (Table 2, entry 9). Nevertheless, poor results were obtained at the substrate concentration of 280 mM (Table 2, entry 10). It suggest that *E. coli*_CtVDH2_NOX is capable of tolerating 240 mM of furfural under the present conditions, which is much higher than those of whole-cell biocatalysts ever reported such as *Acetobacter rancens* IFO3297 (50 mM),[29] *G. oxydans* ATCC 621H (104 mM),[20] *E. coli*_CtVDH1 (50 mM)[15c] and *E. coli*_CtSAPDH (100 mM).[18, 21a]

Table 2. Optimized whole-cell catalytic synthesis of FCA from furfural **4**

Entry	**4** conc. (mM)	Catalyst	Catalyst conc. (g/L)	Time (h)	**4** conv. (%)	FCA yield (%)
1	100	*E. coli*_CtVDH1_NOX	4	12	43 ± 1	35 ± 2
2	100	*E. coli*_CtVDH2_NOX	4	12	>99	93 ± 2
3	100	*E. coli*_CtCALDH2_NOX [a]	4	12	>99	95 ± 3
4	150	*E. coli*_CtVDH2_NOX	4	12	88 ± 3	83 ± 5
5	150	*E. coli*_CtCALDH2_NOX [a]	4	12	68 ± 0	64 ± 2
6	150	*E. coli*_CtVDH2_NOX	11	6	>99	95 ± 3

7	180	*E. coli*_CtVDH2_NOX	11	9	99	93 ± 2
8	240	*E. coli*_CtVDH2_NOX	11	24	64 ± 0	58 ± 0
9	240	*E. coli*_CtVDH2_NOX	22	24	97 ± 0	90 ± 1
10	280	*E. coli*_CtVDH2_NOX	22	24	30 ± 0	27 ± 1

Reaction conditions: 100-280 mM furfural **4**, 4-22 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min; tuning pH to 7 every 2 h using $NaHCO_3$.

[a]: Protein expression was induced at 16 ºC during cell cultivation.

With optimized conditions in hand, high-titer FCA synthesis was conducted with *E. coli*_CtVDH2_NOX, because it could not only considerably improve the volumetric productivity of the bioprocess, but also facilitate the downstream product purification. Considering great toxicity and inhibition caused by high concentrations of substrate, a fed-batch strategy was applied (Figure 3). As shown in Figure 3, approximately 100 mM of furfural was rapidly transformed to FCA within 2.5 h. Besides, furfural **4** was also quickly exhausted in 3.5 h after the first substrate feeding. Approximately 177 mM of FCA was produced from 194 mM furfural **4**, together with 16 mM furfuryl alcohol. The FCA yield and volumetric productivity reached around 91% and 3.3 g/L h, respectively. Nevertheless, the catalytic efficiency of *E. coli*_CtVDH2_NOX sharply decreased after the second substrate feeding, possibly due to partial catalyst inactivation caused by toxic furfural. A period of 7 h was required for complete conversion of 50 mM substrate. Overall, FCA of up to 227 mM was produced within 13 h, along with 19 mM furfuryl alcohol. Total FCA volumetric productivity was about 2.0 g/L h, which is higher than the highest value (1.6 g/L h)[20] in biocatalytic FCA production reported previously.

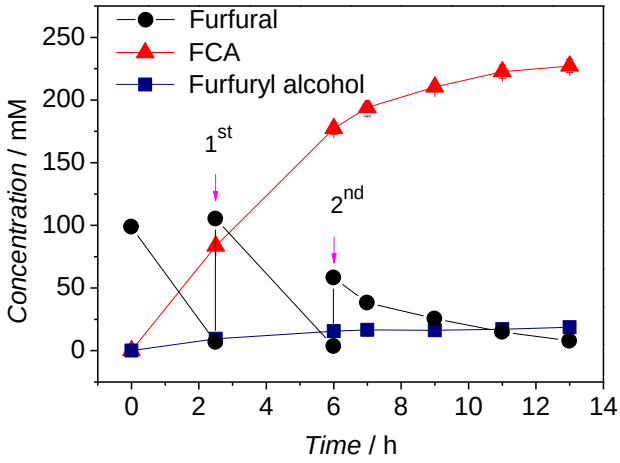

Figure 3. Fed-batch synthesis of FCA by *E. coli*_CtVDH2_NOX. Reaction conditions: 100 mM furfural **4**, 11 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min. Arrows show the feed of furfural (0.2-0.4 mmol) and $NaHCO_3$ (0.4 mmol).

Batch and fed-batch synthesis of MMFCA

As described above, MMFCA is a key starting material for the synthesis of FDCA and TPA. To date, the studies on biocatalytic synthesis of MMFCA remain few.[15b, 18, 21a] Recently, the highest MMFCA volumetric productivity of 1.2 g/L h was reported by our group.[18] To further improve volumetric productivity, optimized synthesis of MMFCA were carried out with cofactor-engineered biocatalysts (Table 3). According to the results in Figure 2, *E. coli*_CtVDH2_NOX and *E. coli*_CtCALDH2_NOX were used as catalysts. As shown in Table 3, *E. coli*_CtVDH2_NOX appeared to excel over *E. coli*_CtCALDH2_NOX for high-titer production of MMFCA, as indicated by comparing MMFCA yields at the substrate concentration of 150 mM (92% vs 79%, entries 3 and 4). The substrate tolerance of *E. coli*_CtVDH2_NOX was greatly improved by simply optimizing cell concentrations. MMF **7** of up to 240 mM could be completely converted within 9 h by 11 g/L of cells, affording MMFCA with a quantitative yield (Table 3, entry 7). Under the same conditions, *E. coli*_CtVDH2_NOX displayed much higher catalytic efficiency in the oxidation of MMF **7** than that in the oxidation of furfural **4**, as evidenced by the reaction periods for completely transforming furans of the same concentrations (e.g., 100 mM, 6 vs 12 h, Table 3, entry

1 vs Table 2, entry 2)

Table 3. Optimized whole-cell catalytic synthesis of MMFCA from MMF **7**

Entry	**7** conc. (mM)	Catalyst	Catalyst conc. (g/L)	Time (h)	**7** conv. (%)	MMFCA yield (%)
1	100	*E. coli*_CtVDH2_NOX	4	6	>99	92 ± 1
2	100	*E. coli*_CtCALDH2_NOX [a]	4	6	98 ± 3	90 ± 4
3	150	*E. coli*_CtVDH2_NOX	4	9	>99	92 ± 3
4	150	*E. coli*_CtCALDH2_NOX [a]	4	9	83 ± 3	79 ± 3
5	200	*E. coli*_CtVDH2_NOX	4	24	70 ± 1	68 ± 1
6	180	*E. coli*_CtVDH2_NOX	11	6	>99	>99
7	240	*E. coli*_CtVDH2_NOX	11	9	>99	99 ± 4
8	280	*E. coli*_CtVDH2_NOX	11	24	47 ± 1	46 ± 2
9	280	*E. coli*_CtVDH2_NOX	22	24	77 ± 2	73 ± 2

Reaction conditions: 100-280 mM MMF **7**, 4-22 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min; tuning pH to 7 every 2 h using $NaHCO_3$.

[a]: Protein expression was induced at 16 ºC during cell cultivation.

Then, fed-batch synthesis of MMFCA was performed with *E. coli*_CtVDH2_NOX (Figure 4). It was found that this biocatalyst maintained a high catalytic activity within 8 h in the synthesis of MMFCA. Around 287 mM of MMFCA was produced from 314 mM of MMF **7**, giving a yield of 91%. Its volumetric productivity was as high as 5.6 g/L h, which is much higher than that a recent value (1.2 g/L h) reported by us with *E. coli*_CtSAPDH.[18] Figure S4 shows that around 1 g of dry cells are obtained from 4.4 g of wet cells upon lyophilization. Based on this equation, the specific productivity of MMFCA in our recent process[18] is about 0.05 g/h per g of cells (CDW). In terms of the specific productivity, therefore, the advantage of the present process using *E. coli*_CtVDH2_NOX appeared to be more significant compared to that with *E. coli*_CtSAPDH (0.5 vs 0.05 g/g h).

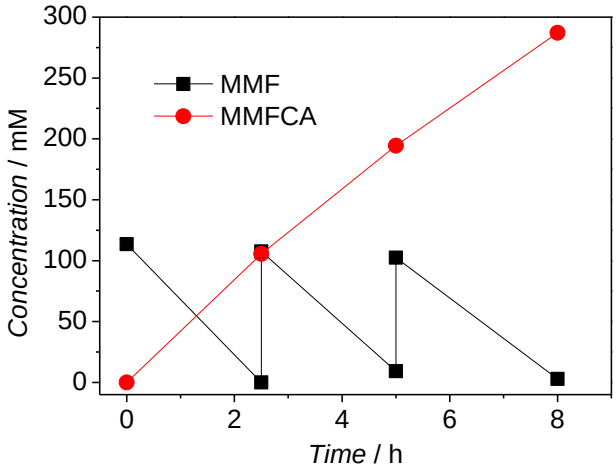

Figure 4. Fed-batch synthesis of MMFCA by *E. coli*_CtVDH2_NOX. Reaction conditions: approximately 100 mM MMF **7**, 11 g/L (CDW) microbial cells, 4 mL K_2HPO_4-NaH_2PO_4 buffer (200 mM, pH 7), 30 °C, 150 r/min. Arrows show the feed of MMF (approximately 0.4 mmol) and $NaHCO_3$ (0.4 mmol).

Conclusions

In summary, high substrate tolerant whole-cell biocatalysts were constructed by a cofactor engineering strategy in this work. These engineered biocatalysts showed a broad substrate scope, and they enabled aerobic oxidation of a group of aromatic aldehydes to target carboxylic acids with good yields. Based on these biocatalysts, a green and sacrificial substrate-free biocatalytic oxidation process was successfully developed for high-titer and high-productivity synthesis of furan carboxylic acids from biobased furans. From sustainable chemistry viewpoint, this bioprocess is advantageous over chemical counterparts, because it uses air as the oxidant, and is totally free of toxic catalysts and solvents. The volumetric productivity up to 5.6 g/L h was obtained in biocatalytic synthesis of furan carboxylic acids. Although improved results such as higher substrate tolerance, increased selectivities and productivities were achieved, unsatisfactory long-term stability of these biocatalysts may be a major obstacle for their large-scale applications in the conversion of toxic furans. Understanding the molecular mechanism of the biocatalyst inactivation caused by furans may be helpful to address this key issue, which is in progress in our laboratory.

Overall, biocatalytic aerobic oxidation may be a promising route for the valorization of biobased furans and make contribution to sustainable synthetic chemistry.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on website.

Strains, plasmids, and primers used in this study, SDS-PAGE analysis of proteins, enzyme activity assay, and HPLC analytic methods and spectra.

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Keywords: aldehyde dehydrogenases; biobased furans; biocatalysis; cofactor engineering; oxidation

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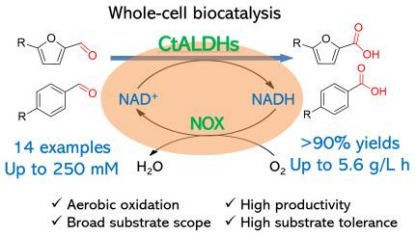
Graphical Abstract



Cofactor-engineered *E. coli* cells proved to be versatile catalysts to produce furan carboxylic acids from toxic biobased furans with high productivities. The introduction of NADH oxidase (NOX) into *E. coli* harboring aldehyde dehydrogenases (ALDHs) significantly promoted intracellular NAD^+ regeneration, thus enhancing ALDH-catalyzed aldehyde oxidation. A group of aromatic aldehydes was efficiently oxidized to target carboxylic acids. More importantly, these engineered whole-cell biocatalysts displayed high substrate tolerance toward toxic furans.