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Concurrent Biocatalytic Oxidation of 5-Hydroxymethylfurfural into 2,5-Furandicarboxylic Acid by Merging Galactose Oxidase with Whole Cells

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Abstract: 2,5-Furandicarboxylic acid (FDCA) is an important monomer for manufacturing biobased plastics. Biocatalysis has been recognized as a sustainable tool in organic synthesis. To date, the efficiencies of most biocatalytic processes toward FDCA remain low. So, it is highly desired to develop efficient processes. In this work, a biocatalytic route toward FDCA was developed by integrating a cell-free extract of galactose oxidase variant M_{3-5} with a whole-cell biocatalyst harboring NAD^+-dependent vanillin dehydrogenases and NADH oxidase, starting from 5-hydroxymethylfurfural. FDCA was produced in a concurrent mode with >90% yields within 36 h at 20 mM substrate concentration. In addition, biocatalytic synthesis of FDCA was performed on a preparative scale, with 78% isolated yield. The present work may lay the foundation for sustainable production of FDCA.

Keywords: aldehyde dehydrogenases; biobased chemicals; biocatalysis; bioplastics; cascade oxidation; oxidases

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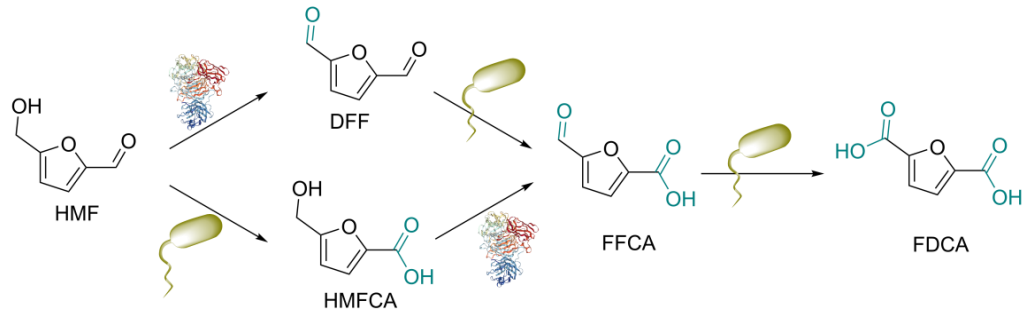
1. Introduction

Recently, the production of biobased chemicals and fuels from renewable and abundant biomass materials such as lignocellulosic biomass has attracted great interest [1–3]. 5-Hydroxymethylfurfural (HMF) obtained via hexose dehydration is considered as a pivotal intermediate bridging the gap between biomass and value-added chemicals/fuels [4]. There exists a primary hydroxyl, a formyl group, and a furan ring in HMF, thus endowing the top-value platform molecule with high chemical reactivity and plasticity. HMF can be converted into a group of valuable chemicals and fuels through typical chemical transformations such as oxidation, reduction, and esterification [5]. Among various HMF derivatives, 2,5-furandicarboxylic acid (FDCA) may be the most important. FDCA is similar in structure to terephthalic acid (TPA), a predominant monomer in polymer and resin manufacture nowadays, with a potential market size of several 100 M EUR; it is presumed as a promising alternative to fossil-based TPA for the production of biobased plastics, including polyesters, polyamides, and polyurethanes [6].

Biocatalysis has emerged as a promising tool for laboratory-scale synthesis as well as industrial manufacture, due to a lot of advantages such as high efficiency, exquisite selectivity, mild reaction conditions, and environmental friendliness [7–9]. In particular, biocatalysis is preferred to chemocatalysis operating under harsh conditions for the upgrading of inherently unstable biobased furans [10,11]. Biocatalytic synthesis of FDCA from HMF and furfural has recently gained considerable attention by using enzymes, cells, and their hybrid systems [11]. Koopman and co-workers presented their pioneering work in biocatalytic FDCA production by *Pseudomonas putida* cells harboring a HMF/furfural oxidoreductase (HmfH) from *Cupriavidus basilensis* HMF14; 30.1 g/L of FDCA was produced from HMF with a 97% yield in a 144-h fed-batch process [12]. A HMF oxidase

(HMFO) was reported by Fraaije and co-workers for the cascade oxidation of HMF into FDCA [13]. Several groups described the FDCA synthesis by combining two enzymes (e.g., galactose oxidase (GO) plus lipase, GO plus aldehyde oxidase, and aryl-alcohol oxidase (AAO) plus unspecific peroxygenase (UPO)) [14–16]. FDCA was produced from HMF with approximately 1.3 g/L h productivity by using *P. putida* expressing HMFO [17] and HmfH/HMF transporter (HmfT1) [18]. Our group developed a recombinant *Escherichia coli* strain coexpressing HmfH and vanillin dehydrogenase (VDH) for HMF oxidation into FDCA [19]. *Raoultella ornithinolytica* isolated from soil was engineered for the FDCA production by Liu and co-workers [20,21]. The combinations of whole-cell biocatalysts and the laccase–TEMPO system were exploited to produce FDCA [22,23]. In addition to HMF, furfural and its derivative 2-furancarboxylic acid (FCA) served as the starting materials for manufacturing FDCA via biocatalytic carboxylation [24–26]. Despite high selectivity, the synthetic efficiencies of the biocatalytic routes reported previously remained low, except for limited cases with approximately 2.6 g/L h productivity [27,28]. Therefore, the development of novel and efficient biocatalytic routes deserves further work.

GO variant $M_{3–5}$ is a versatile oxidase capable of efficiently oxidizing primary and secondary alcohols. This oxidase coupled with other enzymes was used to catalyze biocatalytic cascades to produce FDCA [15,27,28]. Previously, *Comamonas testosteroni* SC1588 was found to enable efficient conversion of furan aldehydes into the corresponding carboxylic acids [29], and NAD^+-dependent VDHs were identified with high activities [30,31]. In this work, an inexpensive and readily prepared GO $M_{3–5}$ cell-free extract (CFE) was integrated with a whole-cell biocatalyst co-expressing VDHs and NADH oxidase (NOX) for concurrent production of FDCA from HMF (Scheme 1).



Scheme 1. Cascade oxidation of HMF into FDCA by combining GO $M_{3–5}$ and whole cells harboring VDHs. DFF, 2,5-diformylfuran; HMFCA, 5-hydroxymethyl-2-furancarboxylic acid; FFCA, 5-formyl-2-furancarboxylic acid.

2. Materials and Methods

2.1. Materials

HMF (95%), 2,5-diformylfuran (DFF, 98%), FDCA (98%), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA, 97%), 5-formyl-2-furancarboxylic acid (FFCA, 98%), and $CuCl_2$ (98%) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Horseradish peroxidase (HRP, RZ > 3.0), β-NADPH (98%), and β-NADH (98%) were from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Catalase (2000–5000 U/mg protein) from bovine liver was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin, and ampicillin were from Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals used in this study were of analytical grade. The recombinant strains *E. coli*_GO $M_{3–5}$, *E. coli*_VDH1_NOX, and *E. coli*_VDH2_NOX were previously constructed [28,31].

2.2. Strain Cultivation

*E. coli*_GO $M_{3–5}$. The recombinant strain *E. coli*_GO $M_{3–5}$ was pre-cultivated in 30 mL Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) containing 50 mg/L kanamycin at 37 °C and 180 r/min overnight. Then, 100 mL LB

medium containing 50 mg/L kanamycin was inoculated with 1 mL of an overnight culture. Cells were cultivated at 37 °C and 180 r/min. When OD_{600} reached 0.6–0.8, IPTG was added to induce enzyme expression at the final concentration of 0.5 mM, followed by incubation at 26 °C and 160 r/min for 48 h. The cells were harvested by centrifugation (8000 r/min, 5 min, 4 °C) and washed twice with 0.85% NaCl solution for the subsequent use.

*E. coli*_VDHs_NOX. The recombinant strains *E. coli*_VDHs_NOX were pre-cultivated in 30 mL LB medium containing 100 mg/L ampicillin at 37 °C and 180 r/min overnight. Then, 100 mL LB medium containing 100 mg/L ampicillin was inoculated with 1 mL of an overnight culture. Cells were cultivated at 37 °C and 180 r/min. When OD_{600} reached 0.6–0.8, IPTG was added to induce enzyme expression at the final concentration of 0.1 mM, followed by incubation 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 for subsequent use.

2.3. Preparation of GO $M_{3–5}$ CFE

The harvested *E. coli*_GO $M_{3–5}$ cells were resuspended in phosphate buffer (50 mM, pH 7) at a cell concentration of 0.1 g/mL (cell wet weight), followed by ultrasonication (350 W, 20 min, 2 s ON, 3 s OFF cycles). The cell lysate was centrifugated at 4 °C (12,000 r/min) for 20 min, affording the supernatant as CFE.

2.4. Enzyme Assay

The GO $M_{3–5}$ CFE activity was spectrophotometrically determined using a UV2550 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) at 25 °C by measuring the guaiacol oxidation at 470 nm (ε of oxidized guaiacol: 12,100 M^{-1} cm^{-1}). The reaction mixture comprised 20 mM HMF, 5 mM guaiacol, 0.05 mg/mL HRP, 1 mM $CuCl_2$, an appropriate amount of GO $M_{3–5}$ CFE, and 2 mL deionized water. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol guaiacol per minute under the above conditions. A representative assay curve was shown in Figure S1.

Enzyme activity calculation was based on the following equation.

$$\text{Enzyme activity (U)} = \text{EW} \times \text{V} \times 10^3/(12{,}100 \times 1)$$

where EW refers to the change in the absorbance within 1 min; V refers to the total volume of the reaction mixture (mL); 12,100 refers to the extinction coefficient of oxidized guaiacol at 470 nm (M^{-1} cm^{-1}); 1 refers to the optical distance (cm).

2.5. Biocatalytic Oxidation of HMF into FDCA

Typically, 2 mL phosphate buffer (200 mM, pH 7) containing 20 mM HMF, 0.5 U/mL GO $M_{3–5}$ CFE, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, and 5 mg/mL *E. coli*_VDH1_NOX (cell wet weight) was incubated at 150 r/min and 30 °C, as shown in Figure S2. Aliquots were withdrawn at specified time intervals from the reaction mixtures and then diluted with the corresponding mobile phase for HPLC analysis. All the experiments were conducted at least in duplicate. The conversions and yields were determined by HPLC, based on the corresponding calibration curves.

2.6. Preparative Synthesis of FDCA

To 20 mL phosphate buffer (200 mM, pH 7), 20 mM HMF, 0.5 U/mL GO $M_{3–5}$, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, and 20 mg/mL *E. coli*_VDH1_NOX (cell wet weight) were added, followed by incubation at 150 r/min and 30 °C for 52 h. After removing cells by centrifugation, the reaction mixture was subject to thermal treatment at 100 °C to remove proteins. Upon protein removal, the supernatant was concentrated and then acidified to pH 1. Excess NaCl was added into the mixture, followed by extraction 3 times with the same volume of ethyl acetate. The organic phases were combined and dried over anhydrous Na_2SO_4 overnight. The crude product of 79 mg was afforded upon solvent evaporation.

2.7. HPLC Analysis

The reaction mixtures were analyzed on a Zorbax Eclipse Plus C18 column (4.6 mm × 250 mm, 5 µm, Agilent, Palo Alto, CA, USA) by using a reversed phase HPLC equipped with a Waters 996 photodiode array detector (Waters, Milford, MA, USA). The mixture of acetonitrile/0.4% $(NH_4)_2SO_4$ solution with pH 3.5 (10:90, *v*/*v*) was used as the mobile phase for quantifying FDCA (264 nm, 4.4 min), FFCA (286 nm, 5.7 min), HMFCA (250 nm, 6.2 min), HMF (283 nm, 9.8 min), and DFF (288 nm, 12.3 min). The column temperature and the flow rate of the mobile phase were 35 °C and 0.6 mL/min, respectively. The representative HPLC chromatogram is shown in Figure S3.

3. Results and Discussion

3.1. Optimization of GO-Catalyzed HMF Oxidation

It has been well known that GO can be considerably activated in the presence of HRP [14,32]. Therefore, the effect of HRP concentrations on the oxidation of HMF by GO $M_{3–5}$ CFE was studied (Figure 1a). It was found that GO $M_{3–5}$ could be greatly activated in the presence of low concentrations of HRP, which is consistent with previous work [33]. After 6 h, HMF was completely converted, affording DFF as the major product (>80% yields). Unexpectedly, the overoxidized product FFCA was produced with 10–20% yields as well. It was previously reported that DFF was produced exclusively in the HMF oxidation by purified GOs [14,27]. So, we presumed that the formation of FFCA might be attributed to the catalytic actions of ALDHs present in CFE. Anyway, the exploitation of GO $M_{3–5}$ CFE may favor the synthesis of FDCA. GO is a copper-containing free radical metalloenzyme [34]. Thus, Cu^{2+} is indispensable for the catalytic activity of the enzyme. Figure 1b shows the effect of the Cu^{2+} concentrations on enzymatic HMF oxidation. The DFF yield (20%) was very low when 0.05 mM Cu^{2+} was present. The increased Cu^{2+} concentrations resulted in the significantly improved DFF yields. For example, DFF was synthesized with 84% yield in the presence of 0.5 mM Cu^{2+}, along with minor FFCA. At 1.0 mM Cu^{2+}, the total yield of DFF and FFCA reached 96%. Despite a high yield, the presence of the high concentration of Cu^{2+} simultaneously led to the reduced catalytic performances of *E. coli*_VDH1_NOX cells (Figure S4), likely due to the negative effect of Cu^{2+} on the activity of VDH1. Therefore, 0.5 mM Cu^{2+} was used in the subsequent studies.

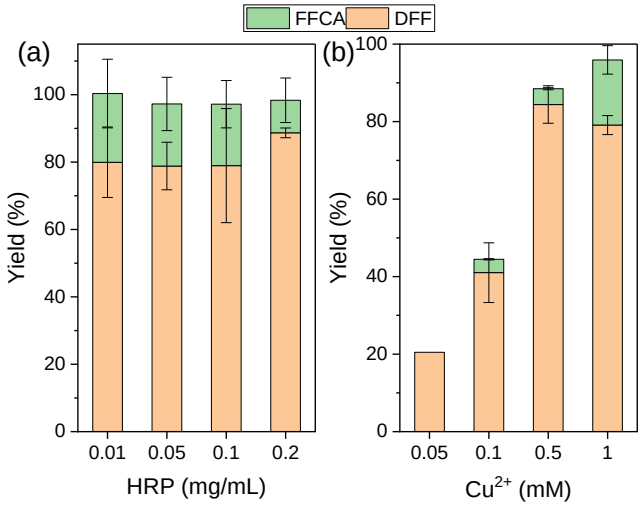


Figure 1. Effect of HRP (**a**) and Cu^{2+} (**b**) concentrations on GO-catalyzed oxidation of HMF. General reaction conditions: 50 mM HMF, 0.5 U/mL GO $M_{3–5}$ CFE, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 1 mM $CuCl_2$, 2 mL phosphate buffer (50 mM, pH 7), 30 °C, 150 r/min, 6 h; (**a**) 0.01–0.2 mg HRP; (**b**) 0.05–1 mM $CuCl_2$.

High substrate loadings are highly desirable because they may result in high catalytic efficiencies as well as high product titers, the latter of which would greatly simplify the downstream product isolation. Therefore, enzymatic oxidation of high concentrations of HMF by GO $M_{3–5}$ CFE was performed (Figure 2). Interestingly, DFF was obtained within 12 h with 82% yield at 100 mM substrate concentration, along with 5% FFCA. Previously, we reported that the DFF yield of 92% was achieved within 96 h by using the wild-type GO of 4 U/mL, starting from 30 mM HMF [14]. Carnell and co-workers reported the utilization of purified GO $M_{3–5}$ of 1 mg/mL for the oxidation of 100 mM HMF, providing access to DFF with an 80% yield in 1 h. So, the GO variant $M_{3–5}$ CFE showed comparable catalytic performance to the purified enzyme, but the preparation of the former was easier. However, the DFF yield was approximately 50% when the substrate concentration was increased to 200 mM.

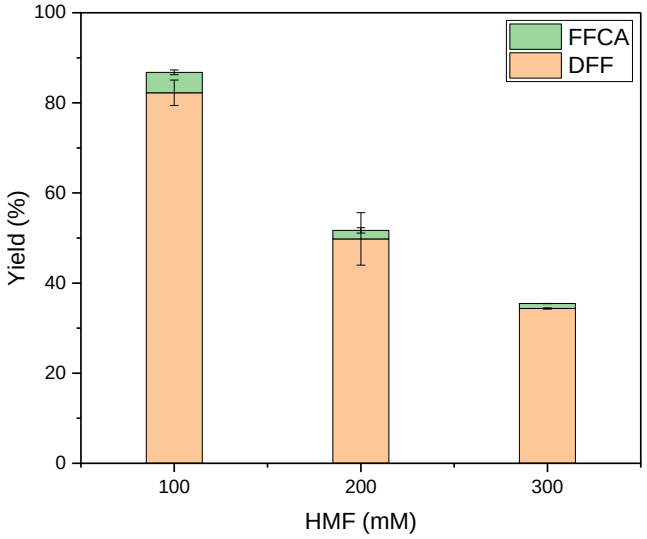


Figure 2. Oxidation of high concentrations of HMF by GO $M_{3–5}$ CFE. Reaction conditions: 100–300 mM HMF, 0.25 U/mL GO $M_{3–5}$ CFE, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, 2 mL phosphate buffer (50 mM, pH 7), 30 °C, 150 r/min, 12 h.

In concurrent HMF oxidation by GO $M_{3–5}$ and whole cells harboring VDHs, HMF may be oxidized into HMFCA by VDHs. So, the oxidation of HMFCA by GO $M_{3–5}$ was examined (Figure S5). It was found that, like HMF, HMFCA was a good substrate of GO $M_{3–5}$, and it was converted into FFCA within 4 h with 90% conversion.

3.2. Whole-Cell Catalytic Oxidation of DFF and FFCA

Previously, VDHs proved to be good catalysts for the oxidation of furan aldehydes into the corresponding carboxylic acids [30,31]. DFF and FFCA are important intermediates in the tandem oxidation of HMF into FDCA (Scheme 1). So, the oxidation of DFF and FFCA by using *E. coli*_VDH1-NOX cells was tested (Figure 3). As shown in Figure 3a, DFF was observed to be a good substrate of VDH1; it was completely converted into FFCA (approximately 83% yield) and FDCA (9%) after 4 h. With the elongation of the reaction period, FFCA was slowly oxidized into FDCA; FDCA was afforded with 24% yield in 12 h. It suggests that FFCA may be a poor substrate of VDH1, evidenced by the fact that the conversion of FFCA was around 78% after 24 h (Figure 3b). Likewise, Matsuda and co-workers reported that *Geotrichum candidum* ALDH showed high activities towards dialdehydes but very low activities towards aldehydic acids [35].

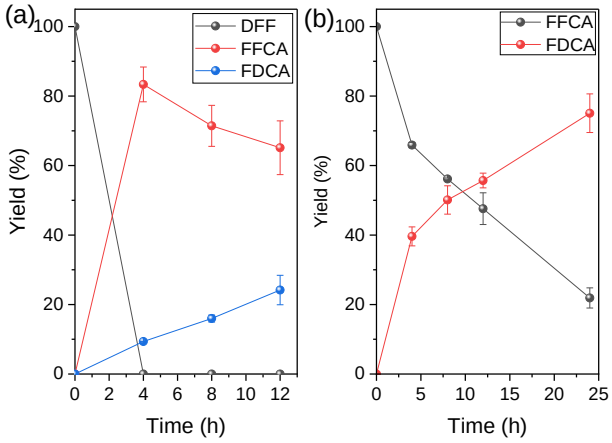


Figure 3. Oxidation of DFF (**a**) and FFCA (**b**) by *E. coli*_VDH1-NOX cells. Reaction conditions: 20 mM DFF/FFCA, 30 mg/mL *E. coli*_VDH1_NOX (cell wet weight), 2 mL phosphate buffer (200 mM, pH 7), 30 °C, 150 r/min.

3.3. Biocatalytic HMF Oxidation into FDCA

Upon establishment of the individual reactions, the cascade oxidation of HMF into FDCA was performed by combining GO M_{3-5} CFE and *E. coli*_VDH1_NOX cells (Figure 4). As show in Figure 4, HMF was completely used up within 6 h. FFCA was produced as a major intermediate (83% yield), along with HMFCA as a minor intermediate. The major intermediate was slowly converted into the targe product FDCA with the elongation of the reaction period. The FDCA yield of 92% was obtained at 36 h. Based on the above results, the FFCA oxidation was the rate-limiting step in the cascade oxidation of HMF into FDCA. To facilitate FFCA oxidation, increasing the cell loading was conducted (Figure S6). Unfortunately, no improved FDCA production was realized. As described above, Cu^{2+} exerted greatly negative effects on the catalytic activities of the whole cells (Figure S4). Therefore, the concurrent production of FDCA was performed in the presence of a lower concentration of Cu^{2+} (0.1 mM) (Figure S7). As shown in Figure S7, HMFCA (about 80%) was formed majorly in 36 h, together with minor FDCA. It might have been due to the remarkedly reduced activity of GO M_{3-5} in the presence of a low concentration of Cu^{2+} (Figure 1b), in spite of improved activities of the whole cells.

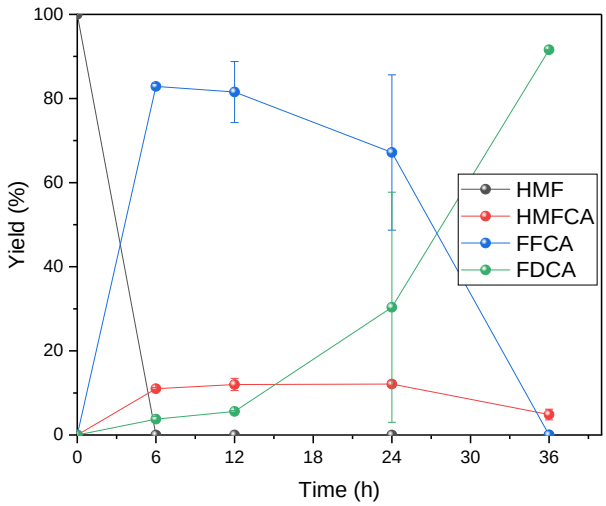


Figure 4. Oxidation of HMF into FDCA by GO M_{3-5} and *E. coli*_VDH1-NOX cells. Reaction conditions: 20 mM HMF, 0.5 U/mL GO M_{3-5} CFE, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, 5 mg/mL *E. coli*_VDH1_NOX (cell wet weight), 2 mL phosphate buffer (200 mM, pH 7), 30 °C, 150 r/min.

An attempt to enhance FDCA production was made by using VDH2 integrated with GO M_{3-5} (Figure 5). Likewise, FFCA was synthesized as a major intermediate, followed by slow conversion into the target product. After 36 h, FDCA was produced with approximately 90% yield, which was comparable to that obtained by VDH1 coupled with GO (Figure 4). The productivity (around 0.08 g/L h) may have been moderate compared to those of the biocatalytic processes reported previously [11]. For example, it was much higher than those with AAOs (coupled with UPO) [16,36,37] and HMFO [13,38] while being considerably lower than those involving GO M_{3-5} [27,28], HmfH [19], and laccase–TEMPO systems [22,23,39]. For example, the FDCA productivities of 0.4–1 g/L h were reported in biocatalytic processes incorporating laccase–TEMPO [22,23,39]. Recent years have seen great advances in the chemically catalytic synthesis of FDCA [40], in spite of some drawbacks, such as harsh reaction conditions and use of environmentally unfriendly catalysts (e.g., metals). For instance, Liguori et al. reported a continuous-flow process based on a heterogenous, resin-supported Pt catalyst, providing access to FDCA in 99% yield at 120 °C within 303 s of residence time; strikingly, the productivity of up to 46 g/L h was achieved [41]. Therefore, biocatalytic production of FDCA is promising but has a long way to go.

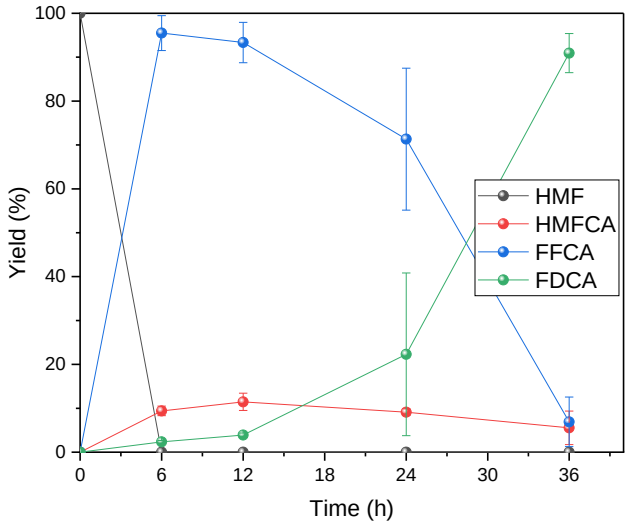


Figure 5. Oxidation of HMF into FDCA by GO M_{3-5} and *E. coli*_VDH2_NOX cells. Reaction conditions: 20 mM HMF, 0.5 U/mL GO M_{3-5} CFE, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, 5 mg/mL *E. coli*_VDH2_NOX (cell wet weight), 2 mL phosphate buffer (200 mM, pH 7), 30 °C, 150 r/min.

3.4. Biocatalytic Preparative Synthesis of FDCA

Biocatalytic preparative synthesis of FDCA was performed on a 20-mL scale by using GO M_{3-5} and *E. coli*_VDH1_NOX cells (Figure 6). Compared to that on a 2-mL scale, the production of FDCA became more sluggish on a 20-mL scale, likely due to the soluble oxygen limitation on a larger scale. It is well known that the solubility of oxygen (approximately 8 mg/L, namely, 0.25 mM) is pretty low in water [42]. After 52 h, approximately 20.5 mM FDCA was produced, with a 94% yield. Upon the reaction and biocatalyst removal, solvent extraction of FDCA by ethyl acetate was performed three times. The organic phases were combined and evaporated, affording a crude product of approximately 76 mg. According to HPLC analysis (Figure S8), the content of FDCA in the crude product was around 70%, along with 5% FFCA. Likely, some protein impurity may have been present in the crude product. The isolated yield of FDCA approached about 78%.

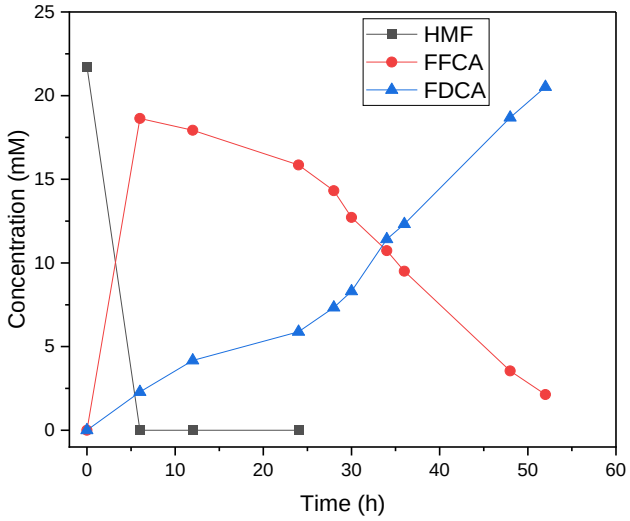


Figure 6. Preparative synthesis of FDCA by GO M_{3-5} and *E. coli*_VDH1_NOX cells. Reaction conditions: approximately 20 mM HMF, 0.5 U/mL GO M_{3-5}, 0.05 mg/mL HRP, 0.1 mg/mL catalase, 0.5 mM $CuCl_2$, 20 mg/mL *E. coli*_VDH1_NOX (cell wet weight), 20 mL phosphate buffer (200 mM, pH 7), 30 °C, 150 r/min.

4. Conclusions

In summary, we developed a biocatalytic route for concurrent production of FDCA from HMF by combining GO M_{3-5} CFE and whole cells harboring VDHs in this work. Use of easily prepared CFE and whole cells as biocatalysts would provide economic benefit compared to purified enzymes. Good FDCA yields (>90%) were obtained at 20 mM substrate concentration within 36 h, with a moderate productivity. As compared to the chemical routes, biocatalysis may be particularly suitable for the oxidation of inherently unstable and highly reactive HMF [10], due to mild reaction conditions and exquisite selectivity. The cost of CFE is apparently much lower than that of the purified enzyme since the former is free of the tedious purification process. Currently, the biocatalytic production was performed in a 2/20-mL scale, with a low substrate loading (20 mM). So, it seemed insignificant to estimate the economic benefit of the present system. In the future, the enzyme immobilization and the biocatalyst recycling will be envisaged in pilot/large-scale processes, which may provide economic benefits as well. Although the use of CFE appears to be relatively cost-effective, the downstream work-up procedure remains complicated. So, the immobilization of GO and cells is of great interest, as it not only stabilizes biocatalysts and allows for the catalyst reuse but also greatly simplifies the downstream process. In the biocatalytic oxidation cascade, the oxidation of FFCA was the rate-determining step. Therefore, the discovery of high-activity ALDHs towards FFCA need further work. Direct conversion of cheap biomass such as fructose and glucose into FDCA is of great interest by chemobiocatalysis [43,44].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11082261/s1, Figure S1: GO assay curve; Figure S2: The picture of the experimental set-up; Figure S3: HPLC chromatogram of the reaction mixture; Figure S4: Effect of 1 mM Cu^{2+} on the HMFCA synthesis by *E. coli*_VDH1_NOX cells; Figure S5: Enzymatic oxidation of HMFCA by GO M_{3-5} CFE; Figure S6: Oxidation of HMF into FDCA by GO M_{3-5} and a high loading of *E. coli*_VDH1-NOX cells; Figure S7: Oxidation of HMF into FDCA by GO M_{3-5} and *E. coli*_VDH1-NOX cells in the presence of 0.1 mM $CuCl_2$; Figure S8: HPLC analysis of the crude product.

Author Contributions: Investigation, F.-F.Z. and J.-P.W.; Methodology, F.-F.Z. and J.-P.W.; Bioresources, Z.-J.Z.; Conceptualization, N.L.; Writing—Original Draft Preparation, N.L.; Writing—Review and Editing, J.-P.W., M.-H.Z. and N.L.; Funding Acquisition, M.-H.Z. and N.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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