Biocatalysis

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Enzyme-Catalyzed Oxidation of 5-Hydroxymethylfurfural to Furan-2,5-dicarboxylic Acid**

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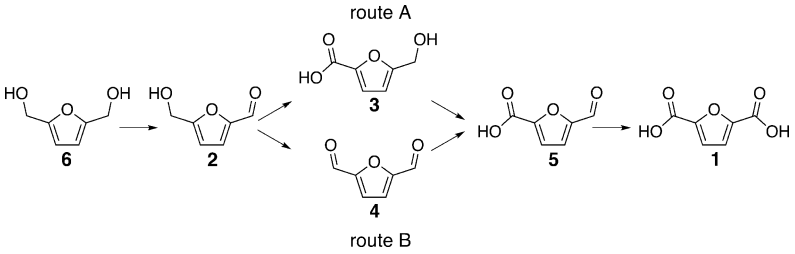
Abstract: *Furan-2,5-dicarboxylic acid (FDCA) is a biobased platform chemical for the production of polymers. In the past few years, numerous multistep chemical routes have been reported on the synthesis of FDCA by oxidation of 5-hydroxymethylfurfural (HMF). Recently we identified an FAD-dependent enzyme which is active towards HMF and related compounds. This oxidase has the remarkable capability of oxidizing [5-(hydroxymethyl)furan-2-yl]methanol to FDCA, a reaction involving four consecutive oxidations. The oxidase can produce FDCA from HMF with high yield at ambient temperature and pressure. Examination of the underlying mechanism shows that the oxidase acts on alcohol groups only and depends on the hydration of aldehydes for the oxidation reaction required to form FDCA.*

The increasing interest in renewable, biobased polymers has resulted in the quest for new synthesis routes for the production of polymer building blocks. One promising biobased polymer is polyethylene furanoate (PEF), a polyester which is comparable to the well-known petroleum-based polyethylene terephthalate (PET).[1,2] PEF is the esterification product of ethane-1,2-diol and furan-2,5-dicarboxylic acid (FDCA; **1**). Although **1** is not commonly found in nature, it can be derived from abundant C6 sugars like fructose and glucose. In a two-step process, C6 sugars are dehydrated to 5-hydroxymethylfurfural (HMF; **2**) which can subsequently be converted into **1** through three oxidative steps.[3] Because **1** is often mentioned as a valuable building block,[4] numerous chemical pathways have been proposed for this oxidative process. Methods range from the use of stoichiometric oxidants,[5] to homogeneous metal salts like cobalt and manganese,[6] to heterologous metal catalysts such as Pd/Au or porphyrin-based porous organic polymers loaded with Fe^{III}.[7,8] All of these methods are typically performed at elevated temperatures and pressure. Additionally, they produce a mixture of **1**, intermediate oxidation products, and side products (Scheme 1).

In contrast to the chemical synthesis, the biochemical production of **1** from **2** is less well established. To date, a fermentative process using *Pseudomonas putida* as a host organism is the only described example.[9] This process relies on the activities of both oxidases and dehydrogenases. Biocatalytic production of **1** by a single enzyme has thus far been impossible because of the absence of an HMF oxidase and the complexity of the oxidation. Most enzymes are restricted to either alcohol or aldehyde oxidations, while the full oxidation of **2** to **1** requires the enzyme to act on both alcohol and aldehyde groups. Because the production of **1** from **2** entails three consecutive oxidation steps, all reaction intermediates would have to be accepted as substrates as well. On the whole, the enzymatic production of **1** is far from straightforward.



Scheme 1. The oxidation of [5-(hydroxymethyl)furan-2-yl]methanol (**6**) and **2** into the diacid **1** can proceed by two routes. Route A: the aldehyde group of **2** is oxidized to form a carboxylic acid. Route B: the alcohol group of **2** is oxidized to yield the dialdehyde **4**. HMFO exclusively uses route B. Each step is a two-electron oxidation in which one equivalent of O_2 is consumed and one equivalent of H_2O_2 is formed by the enzyme.

Recently, we identified an FAD-dependent oxidase of the glucose-methanol-choline oxidoreductase (GMC) family, and it was named HMF oxidase (HMFO) and is the first enzyme with proven oxidase activity on **2**.[10] The activity of HMFO is not restricted to **2**, because the enzyme has a relatively broad substrate scope and accepts many different aromatic alcohols and aldehydes related to **2**. This makes the enzyme a good candidate for the biocatalytic production of **1**. The oxidation from **2** to **1** can occur by two routes (Scheme 1). In the first route, the aldehyde group of **2** is oxidized to the carboxylic acid, thus yielding 5-(hydroxymethyl)furan-2-carboxylic acid (**3**). A second oxidation yields 5-formylfuran-2-carboxylic acid (**5**) and this will ultimately be converted into **1**. In the second route, the alcohol group of HMF is oxidized to the

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corresponding aldehyde, thus yielding furan-2,5-dicarbaldehyde (**4**), followed by additional oxidations to **5** and **1**. Because HMFO was shown to be active towards both **3** and **4**, both routes would be feasible for HMFO.

To gain more insight into the catalytic properties of HMFO, the conversion of **2** and its derivatives was monitored over time. Reactions were started upon addition of the enzyme and samples were taken during conversion. The reaction was terminated by heating the sample for five minutes at 70 °C to denature the enzyme. All samples were analyzed by HPLC. Using **2** as a substrate, the oxidation products **4**, **5**, and **1** were detected during the conversion. The compound **4** was found to be the main intermediate in the first hours of the reaction, whereas increasing amounts of **5** and **1** appeared from the 4 to 15 hour time point (Table 1 and see Tables S1 and S2 in the Supporting Information). The exclusive formation of **4** shows that route B is the preferred oxidation pathway and route A is not used by HMFO (Scheme 1).

Table 1: Oxidation of furans to **1** at 25 °C in a 100 mM potassium phosphate buffer of pH 7.0. The reaction time was 15 h, unless stated otherwise.

Entry	Substrate	[mM]	HMFO [μM]	FAD [μM]	Conv. [%][a]	Yield [%] FDCA
1	**6**	2	1	–	100	4.4 ± 0.2
2	**2**	2	1	–	100	5.0 ± 0.1
3	**2**	2	1	10	100	6.1 ± 0.5
4	**2**	4	20	20	100	95 ± 1.7[b]
5	**4**	2	1	–	100	5.9 ± 0.1
6	**3**	2	1	–	99	6.1 ± 0.6
7	**5**	2	1	–	7.4	7.4 ± 0.1

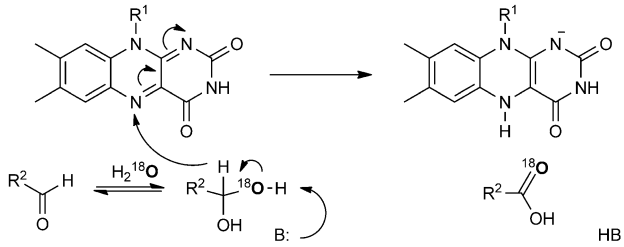
[a] 100% conversion means the substrate is fully converted. [b] The reaction time was 24 h.

To determine whether the preference for route B is valid for other substrates as well, we expanded the analysis to the phenylic analogues of the examined furans. By using the phenylic analogue of **2**, 4-(hydroxymethyl)benzaldehyde (**7**), as the substrate, the conversion was monitored over time. Similar to the experiments on the furanic compounds, terephthaldehyde (**8**), 4-formylbenzoic acid (**9**), and terephthalic acid (**10**) were formed. The phenylic analogue of **3**, 4-(hydroxymethyl)benzoic acid (**11**), was again not formed as an intermediate (see Table S6, S7, and S12 in the Supporting Information).

These findings imply that HMFO oxidizes the alcohol group of **2** and **7** first, rather than the aldehyde substituent. This forces the enzyme to perform an aldehyde oxidation as the next step, as **4** and **8** do not contain an alcohol group. The good activity towards the furanic and phenylic dialdehydes (**4** and **8**) is surprising because HMFO does not react with the aldehyde groups of **2** and **7**. The subsequent oxidation of aldehydes **5** and **9** into the final product is much less efficient as evidenced by the presence of these oxidation intermediates as main products when relatively little enzyme was used (Table 1, entries 1–3 and 5–7). To optimize the yield of **1**, the pH value, temperature, and enzyme concentration were varied. In addition, FAD was added to prevent the inactivation of HMFO because of cofactor dissociation. HMFO relies on a noncovalently bound FAD cofactor for catalysis and it can dissociate from the enzyme, thus rendering it inactive. The effect of additional FAD is marginal but clear, thus increasing the yield of **1** from 5% to 6.1% (Table 1, entries 2 and 3). High yields of **1** were obtained using a biocatalyst/substrate ratio of 1:200. A reaction with 20 μM HMFO and 4 mM **2** produces a yield of 95% of **1** after 24 hours at ambient temperature and pressure (Table 1, entry 4). It is worth noting that the residual activity of HMFO was close to 100% after 24 hours of incubation. This data shows that the biocatalyst is rather robust and suitable for prolonged incubations.

The oxidation route (Scheme 1) raises the question as to why HMFO is an efficient catalysis for oxidation of aldehydes like **4** and **8**, but leaves the aldehyde groups of **2** and its phenylic analogue **7** unaffected. Alcohol oxidation by FAD-dependent oxidases is typically initiated by the abstraction of a proton from the alcohol group by an active-site base, and is followed by a hydride transfer from the α-carbon atom of the alcohol to the FAD cofactor.[11] With aldehydes, such a mechanism is not possible and may be the reason why most FAD-dependent oxidases are not capable of oxidizing aldehydes.[12]

However, several oxidases have been reported to perform aldehyde oxidation reactions. In all these cases, the aldehyde itself is not oxidized, but the hydrated *gem*-diol form is converted by the enzyme .[13–15] To investigate the mechanism of oxidation by HMFO, ^{18}O-labeled water was used during catalysis (Scheme 2). When the hydrated aldehyde is oxidized to a carboxylic acid by HMFO, one water-derived oxygen atom (^{18}O) will be incorporated. In contrast, if the oxidation is spontaneous, ^{16}O from molecular oxygen will be incorporated. To distinguish between these reactions, the oxidation product of the readily hydrated 4-nitrobenzylaldehyde,[14] formed by HMFO, was analyzed by LC-MS. In unlabeled water, the formed product has the expected mass of 166 (MS in negative mode), thus corresponding to 4-nitrobenzoic acid. A similar experiment, performed in 30% $H_2{}^{18}O$, gives additional mass peaks of *M*+2 and *M*+4 (Figure 1). This shows that the inserted oxygen originates from the hydration of the aldehyde and HMFO subsequently oxidizes the hydration product to the carboxylic product.



Scheme 2. Hydration and oxidation of an aldehyde. The formed *gem*-diol is oxidized by the enzyme to form the carboxylic acid. The presence of $H_2{}^{18}O$ in the reaction will lead to the formation of a product with *m*/*z* values of *M*+2 and *M*+4.

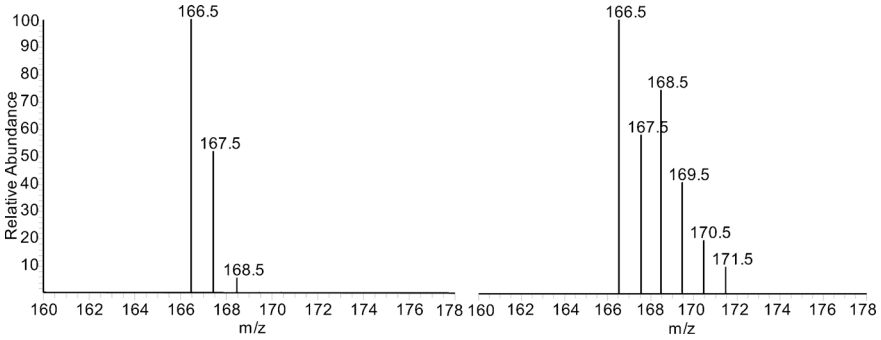
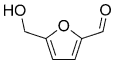
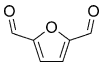
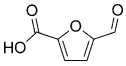
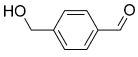
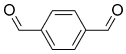
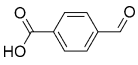


Figure 1. APCI-MS (negative mode) of HMFO-formed 4-nitrobenzoic acid. The spectrum on the left shows the product formed in H_2O, and the spectrum on the right shows the increased masses of 168 and 170 (M+2 and +4 respectively) when oxidation of 4-nitrobenzaldehyde was performed in 30% $H_2{}^{18}O$.

To investigate the degree of hydration of the aldehydes involved in the production of **1**, all aldehydes were analyzed by ^{1}H NMR spectroscopy. Reference spectra of the aldehydes were recorded in deuterated dimethylsulfoxide or chloroform. Dissolving the aldehydes in D_2O provides insight into the hydration state. Because the hydration is influenced by pH,[16] a buffer of 100 mM potassium phosphate of pH 7.0 was used, thus mimicking the conditions during biocatalytic conversions. The proton on the carbonyl carbon atom of the aldehyde typically displays a chemical shift of $\delta = 9$ ppm. Upon hydration, the same proton appears around $\delta = 6$ ppm. To determine the ratio of aldehyde to *gem*-diol, the ratio of the integrated area of these two peaks is used. From the data in Table 2, it becomes clear that the degree of hydration of the different aldehydes is dependent on the substituents on the ring. The aldehyde groups of **4** and **8** are easily hydrated, but when the substrate also has a carboxylic acid substituent, the hydration of the aldehyde groups can be reduced by a factor of 5–20, as seen for **9** and **5**.

Table 2: The degree of hydration and the kinetic parameters for HFMO of all studied aldehydes at 25 °C, pH 7.0.

Entry	Substrate		*gem*-Diol [%]	k_{cat} [s^{-1}][a]	K_M [mM][a]	k_{cat}/K_M [s^{-1} mM^{-1}]
1	**2**		0.0	9.9	1.4	7.0
2	**4**		29	1.6	1.7	0.94
3	**5**		1.8	n.d.[b]	n.d.	< 0.01
4	**7**		0.4	8.6	0.15	57
5	**8**		8.4	1.3	0.085	15
6	**9**		1.9	n.d.	n.d.	0.028

[a] The kinetic parameters for **2**, **4**, and **5** have been described elsewhere.[10] n. d. = not determined.

To gain more insight into the relation between aldehyde hydration and oxidation by HMFO, the steady-state kinetic parameters of the aldehydes are compared to the degree of hydration. The kinetic parameters of **2**, **4**, and **5** were determined previously and the parameters for **7**, **8**, and **9** were determined by the same method for this study.[10] An interesting trend is observed when the kinetic parameters and the degree of hydration of each HMFO substrate are compared. The catalytic efficiency of HMFO on **4** and **8** is high as can be seen from their respective k_{cat}/K_M values (Table 2, entries 2 and 5) and this coincides with their relatively high degree of hydration. The compounds **5** and **9**, in contrast, have a poor catalytic efficiency which correlates with the low degree of hydration for these aldehydes (Table 2, entries 3 and 6). The compounds **2** and **7** also show a very low degree of hydration, but are efficiently oxidized (Table 2, entries 1 and 4). This conversion is because in these cases the alcohol moiety of these compounds is oxidized and not the aldehyde. Furthermore, one should realize that the K_M values in Table 2 have been determined based on the concentration of substrate used. Because the enzyme seems to be only active on the hydrated forms of DFF and terephthalate, the actual substrate concentration is in fact lower and as a result the K_M values are overestimated. In the case of **4**, it indicates that HMFO displays a higher affinity (K_M) when compared with **2**, thus suggesting that the additional hydroxy group of the *gem*-diol is favorable for substrate recognition. The same holds for **8**, in which the effect is even more pronounced.

The results above show that, by starting with **2** as a substrate the alcohol oxidation product **4** will be formed (route B). The aldehyde group of **2** cannot be oxidized because it is not present in its hydrated form. A significant fraction of **4** is hydrated, and therefore it is readily oxidized to **5**. Only a minor fraction of **5** is in the hydrated form, and could explain why the conversion of **5** was quite low and becomes the compound accumulated during the reaction. The phenylic substrates behave similarly to their furanic analogues. Hence, the enzyme takes the analogous oxidation route and again the oxidation of **9** is relatively slow.

From these studies, it becomes clear that HMFO is a true alcohol oxidase. Activity on **4** and **5** is only possible upon hydration of the aldehyde group into the corresponding *gem*-diol, which is subsequently oxidized by HMFO (Scheme 2).

The production of **1** is mostly studied with **2** as the starting material. For an alcohol oxidase, the reduced form of **2**, [5-(hydroxymethyl)furan-2-yl]methanol (**6**), could also be used as starting material. In fact, we found that **6** and its phenylic analogue 4-(hydroxymethyl)phenyl]methanol (**12**) are the substrates with the highest k_{cat} values for HMFO determined thus far (22 s^{-1} for **6** and 21 s^{-1} for **12**).[10] To explore the oxidative catalytic power of HMFO, reactions on both **6** and **12** were performed and analyzed by HPLC. Starting from both substrates, the corresponding diacids **1** and terephthalic acid (**10**) were formed. This means that HMFO can perform four consecutive oxidation steps. Most oxidases perform only

a single oxidation[17,18] and only a few enzymes have been reported to perform two sequential oxidations.[19,20] In this light, the finding that HMFO is capable of four sequential oxidations is quite remarkable.

The flavoprotein oxidase HMFO is the first enzyme described to produce **1** from **2**. A great benefit of oxidase biocatalysts such as HMFO is their independence of expensive cofactors, as they use molecular oxygen as terminal electron acceptor. Many chemical methods for the production of **1** use increased air or oxygen pressure, ranging from 0.3 to 7 MPa, to facilitate the reaction.[6,21] By using HMFO, the reaction can be performed at ambient pressure (0.1 MPa). In addition, the reaction can be performed at 25 °C, whereas chemical routes typically require temperatures from 60 °C up to 130 °C.[7,8,22] To obtain yields above 95 % for **1**, prolonged reaction times were required for our enzyme-based reaction. The reaction time of 24 hours is mainly a result of the poor reactivity of HMFO on the last intermediate **5**. Because the enzyme is quite stable, the prolonged reaction times do not pose a problem.

With the discovery of HMFO, we believe to have found a good biocatalytic alternative for the production of FDCA (**1**) from HMF (**2**). The complete oxidation of **2** to **1** by a single (bio)catalyst is challenging as it involves three consecutive oxidations. HMFO is able to perform this formidable task by acting on the hydrated aldehyde intermediates. Remarkable is the oxidation of **6**, in which the oxidase performs four sequential oxidations, and to the best of our knowledge it is a novelty for biocatalytic oxidations.

Experimental Section

HMFO was expressed in *E. coli* BL21 (DE3) and purified using affinity chromatography as described before.[10] Kinetic parameters were determined using O_2 depletion measurements as described before.[10] Analysis of **2** and **7**, and their oxidation products were performed on a HPLC using a Zorbax Eclipse XDB-C8 column (Agilent) or a Gemini-NX C18 column (Phenomenex), respectively. LC-MS was performed on a Surveyor LC with LCQ Fleet MS (Thermo Finnigan) using an Alltima HP C18 column (Grace) and APCI ionization. ^{1}H NMR spectra of the aldehydes were recorded on a Varian Gemini NMR spectrometer of 300 MHz at 25 °C.

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