

Multistep Biooxidation of 5-(Hydroxymethyl)furfural to 2,5-Furandicarboxylic Acid with H₂O₂ by Unspecific Peroxygenases

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5-(Hydroxymethyl)furfural (HMF) is a key platform chemical derived from renewable biomass sources, holding great potential as starting material for the synthesis of valuable compounds, thereby replacing petrochemical-derived counterparts. Among these valorised compounds, 2,5-furandicarboxylic acid (FDCA) has emerged as a versatile building block. Here we demonstrate the biocatalytic synthesis of FDCA from HMF via a one-pot three-step oxidative cascade performed via two operative steps under mild reaction conditions employing two unspecific peroxygenases (UPOs) using hydrogen peroxide as the only oxidant. The challenge of HMF oxidation by UPOs is

the chemoselectivity of the first step, as one of the two possible oxidation products is only a poor substrate for further oxidation. The unspecific peroxygenase from *Marasmius oreades* (*MorUPO*) was found to oxidize 100 mM of HMF to 5-formyl-2-furoic acid (FFCA) with 95% chemoselectivity. In the sequential one-pot cascade employing *MorUPO* (TON up to 13535) and the UPO from *Agrocybe aegerita* (*AaeUPO*, TON up to 7079), 100 mM of HMF were oxidized to FDCA reaching up to 99% conversion and yielding 861 mg isolated pure crystalline FDCA, presenting the first example of a gram scale biocatalytic synthesis of FDCA involving UPOs.

Introduction

The increasing demand for sustainable and environmentally friendly alternatives to conventional chemical processes has fuelled the exploration of biocatalytic strategies for the synthesis of high-value chemicals from renewable biomass feedstocks.^[1] Among these, the biocatalytic oxidation of biomass derived 5-(hydroxymethyl)furfural (HMF) to 2,5-furandicarboxylic acid (FDCA) represents a promising pathway to develop

a circular bioeconomy.^[2] FDCA is a versatile precursor for the synthesis of bio-based polymers, including polyethylene furanoate (PEF), a viable alternative to traditional polyethylene terephthalate (PET). Similar to PET, PEF lacks inherent biodegradability but is reported to have enhanced susceptibility to enzymatic hydrolysis^[3] and efforts have been made to design biodegradable copolymers including FDCA as a building block.^[4] The production of FDCA via catalytic oxidation of HMF has been extensively studied and excellent reviews are available in the literature which cover hetero- and homogenous catalysis^[5] as well as biocatalysis.^[6,7-13] Besides potential environmental benefits when using a bioderived and biodegradable catalyst at ambient conditions, additionally undesired condensation reactions of HMF taking place already at moderately elevated temperatures may be avoided.^[7,14] Hitherto, molecular oxygen dependent oxidases have predominantly been used for HMF oxidation producing H₂O₂ as a byproduct.^[8-10,15] On the other hand, advantages of recently identified unspecific peroxygenases (UPOs)^[16,17] are that they are robust, scalable^[18] and solely rely on H₂O₂ as the oxygen source and terminal electron acceptor, generating H₂O as the only byproduct.^[19] To date, only the UPO from *Agrocybe aegerita* (*AaeUPO*) was applied in combination with an aryl alcohol oxidase from *Pleurotus eryngii* (*PeAAO*) for converting HMF to FDCA.^[11-13] However, the reactions were performed on analytical scale and the additional oxidase was required as *AaeUPO* alone is not performing all three consecutive oxidation steps essential for the transformation of HMF to FDCA, thereby requiring two oxidants namely molecular oxygen and H₂O₂.

In this work, we screened a panel of commercially available UPOs for their potential to oxidize HMF and propose a scalable

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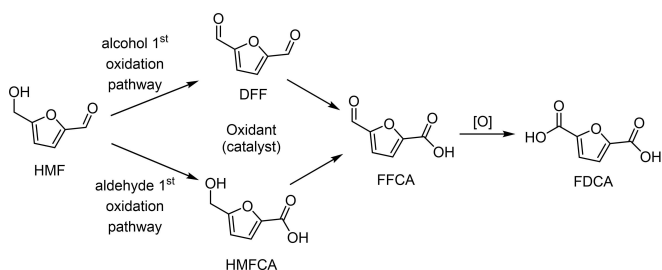
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one pot cascade employing UPOs only for the three-step oxidation and subsequent isolation of FDCA.



Scheme 1. General scheme for the oxidation of HMF to FDCA.

Results and Discussion

HMF is oxidized to FDCA via three oxidation steps, whereby for the first step two options are possible (Scheme 1). The initial oxidation can occur either at the alcohol moiety giving the dialdehyde DFF (alcohol 1st oxidation pathway), or at the aldehyde moiety of HMF yielding (hydroxymethyl)furan carboxylic acid (HMFCFA) (aldehyde 1st pathway). The oxidation of both compounds leads to FFCA and subsequently to FDCA.

UPO Screening & Reaction Engineering with HspUPO

Initially a panel of UPOs was screened for their potential to oxidize HMF (Table 1). In these experiments H₂O₂ was supplied

Table 1. UPO Screening for the oxidation of HMF.^[a]

UPO	Conversion [%]	Molar product distribution [%]			
<i>Aae</i> UPO	35	59	37	4	n.d.
<i>Abr</i> UPO	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Hsp</i> UPO	> 99	n.d.	26	70	4
<i>Pan</i> UPO	70	17	43	40	n.d.
<i>Dsp</i> UPO-I	24	13	82	5	n.d.
<i>Atu</i> UPO	2	53	45	2	n.d.
<i>Ani</i> UPO	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Alu</i> UPO	6	55	40	5	n.d.
<i>Hsp</i> UPO-II	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Dsp</i> UPO-II	5	85	10	5	n.d.
<i>Aac</i> UPO	3	60	38	2	n.d.
<i>Gma</i> UPO-II	42	25	54	22	n.d.
<i>Cab</i> UPO-III	52	79	14	8	n.d.
<i>Lsp</i> UPO	25	60	35	5	n.d.
<i>Glu</i> UPO	6	58	15	27	n.d.
<i>Aps</i> UPO	> 99	33	1	66	n.d.
<i>Abo</i> UPO	23	96	1	3	n.d.
<i>Mfu</i> UPO	2	35	35	30	n.d.
<i>Psp</i> UPO	11	79	8	13	n.d.
<i>Sni</i> UPO	62	40	39	21	n.d.
<i>Cab</i> UPO-V	31	29	68	4	n.d.
<i>Cab</i> UPO-VII	2	88	3	9	n.d.
<i>Mor</i> UPO	> 99	36	7	56	n.d.
Neg. Ctrl. ^[b]	n.d.	n.d.	n.d.	n.d.	n.d.

^[a] Conditions: HMF (10 mM), UPO (10 μM), KPi 0.1 M pH 7.4, room temperature, 48 h. H₂O₂ (33 mM, 0.69 mM h⁻¹) was continuously supplied via a syringe pump. Conversion & molar product ratio were determined on HPLC by external calibration with commercial reference material. ^[b] Negative control reaction (UPO omitted). n.d. = not detected.

via continuous addition of 3.3 equivalents representing a slight stoichiometric excess considering all three oxidation steps. The continuous addition of H_2O_2 proved superior to stepwise addition most likely because the concentration of H_2O_2 was staying low due to consumption of the oxidant as long as the reaction proceeded. Three UPOs, namely *HspUPO*,^[20] *ApsUPO*^[17] and *MorUPO*^[17] were able to consume all HMF under the reaction conditions employed. Of these three UPOs, *HspUPO* showed the formation of low amounts of the desired dicarboxylic acid FDCA qualifying it as the first UPO capable of executing all three consecutive oxidation steps producing FDCA from HMF at these conditions. This substrate flexibility aligns with the wide range of substrates that *HspUPO* has been reported to oxidize, including primary and secondary alcohols along with aldehydes.^[20]

Subsequently, attempts were undertaken to improve the conversion to FDCA at 10 mM substrate concentration by investigating several reaction parameters of the biotransformation with *HspUPO*. Although *HspUPO* is reported to be thermotolerant,^[20] raising the reaction temperature from room temperature (21–23 °C) to 30 °C turned out to be detrimental leading to a significant drop of the conversion (see SI). Increasing the amount/concentration of H_2O_2 to 6.6 or 9.9 equivalents also did not lead to increased productivity. Changing the reaction medium from phosphate buffer to commonly used buffers (TRIS, tricine, HEPES, glycylglycine and TAPS) showed no benefit and in general only very little effect (see SI). Interestingly, it was noted that by varying the cosolvents (5% v/v), the chemoselectivity in the first oxidation step could be tuned (Figure 1). This may be explained by cosolvent induced conformational change in the enzyme that alter the substrate binding causing a change of selectivity, a phenomenon previously described.^[21]

In most cases (except for EtOH, *n*-PrOH and 1,4-dioxane, where activity was diminished) the amount of HMFCa in the molar product ratio was reduced compared to the cosolvent-free biotransformation, however, at the cost of reduced activity.

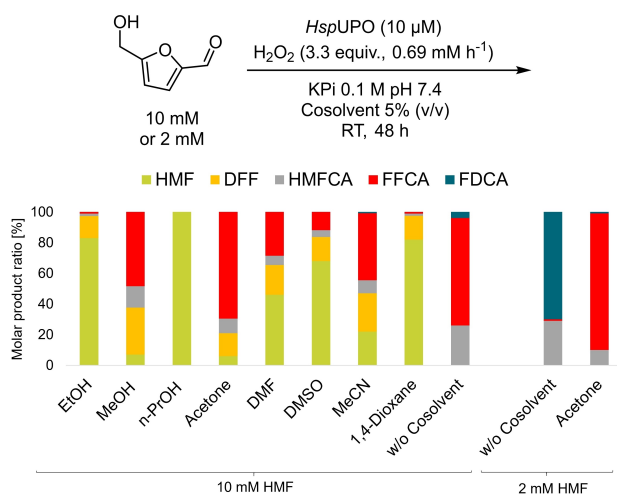


Figure 1. Screening of various cosolvents for the oxidation of HMF by *HspUPO* and lowering the substrate concentration to 2 mM. Conditions: HMF (2 or 10 mM), *HspUPO* (10 μM), KPi 0.1 M pH 7.4, room temperature.

It was found that HMFCa is almost a 'dead-end' product in the oxidation pathway, as HMFCa is only very slowly converted by *HspUPO* as demonstrated in a separate experiment (~5% within 24 h, Table S6). Inhibition by HMFCa was excluded as *HspUPO* reached completion in the transformation of 50 mM *rac*-1-phenylethanol in the presence of 3 mM HMFCa (see SI). In fact, none of the UPOs in our set of enzymes converted efficiently HMFCa, thwarting a potential cascade between *HspUPO* and another UPO that would accept HMFCa as a substrate.

When the HMF concentration was reduced to 2 mM, i.e. the biocatalyst/substrate ratio was increased, HMF was oxidized to FDCA by *HspUPO* with 63% FDCA formation, leaving HMFCa as by-product. When adding 5% v/v acetone to improve the chemoselectivity in the first oxidation step, only trace amounts of FDCA were obtained in the reaction with 2 mM HMF, consistent with the observed reduced activity in the presence of this cosolvent (Figure 1).

Since the UPOs investigated were not able to oxidize HMFCa efficiently (see Table S6), but other types of enzymes like the 5-(hydroxymethyl)furfural oxidase (HMFO) have been described to do so including all other oxidation steps of the sequence,^[10] a cascade combining UPOs and HMFO and variants thereof were considered. However, testing such cascades showed no added benefit when comparing the HMFO/*HspUPO* cascade with the control reaction where just HMFO was used. In fact, experiments revealed that increasing the UPO concentration in the cascade reaction led to decreasing conversions, suggesting that HMFO is incompatible with the unspecific peroxygenases tested (Data is detailed in the supporting information).

Structural Investigations

As the chemoselectivity in the first oxidation step was identified to be crucial to reach high formation of FDCA and not to reduce the outcome by the dead-end pathway (aldehyde 1st oxidation pathway) giving HMFCa as an undesired co-product, we investigated the enzyme structure to identify possible structural factors influencing the chemoselectivity. Docking studies with HMF as the ligand and selected UPOs were performed (Figure 2) whereby *ApsUPO* and *MorUPO* were chosen representing UPOs catalysing the HMF oxidation preferentially via the 'alcohol 1st oxidation pathway' generating mainly DFF. For comparison *DspUPO*-I was selected which performed HMF oxidation preferentially via the 'aldehyde 1st oxidation pathway' generating primarily HMFCa (Table 1). For comparison also *HspUPO* was chosen as a moderately selective enzyme. HMF was docked in its aldehyde (non-hydrated) form since the hydration equilibrium is on the aldehyde side.^[12] The docking modes with the lowest energy of binding correctly resembled the experimentally observed chemoselectivity (Figure 2). Thus, the alcohol moiety of the docked HMF ligand is closer to the ferryl oxygen of compound I ($\text{O}_{\text{Cpd-I}}$) in *MorUPO*, *ApsUPO* and also *HspUPO*, while the aldehyde moiety is closer in *DspUPO*-I. Special attention was paid to minimize bias in the docking process by varying the docking parameters, which includes a)

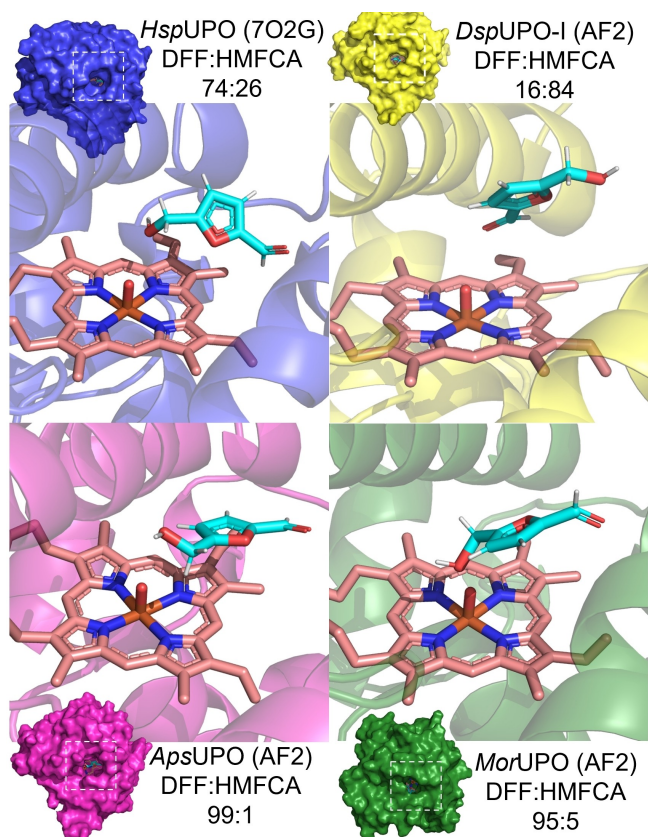


Figure 2. Molecular docking of HMF into UPOs with different chemoselectivity in the first oxidation step (ratio refers to molar ratio of DFF:HMFCFA). Docking poses with lowest energies are displayed. AF2 = Alpha-Fold2 homology model.

different input conformations of the HMF ligand, i.e. docking three of the most stable conformations in aqueous system as determined in a dedicated study from literature,^[22] b) modelling the heme cofactor as Cpd-I or in its native state, c) introducing flexibility into active site, which means freeing contacting receptor residues and d) adjusting the size and rotation of the simulation cell.

Nevertheless, it is important to note that independent of the docking parameters used the analysis of binding energy of relevant clusters generated by the VINA docking algorithm (differing at least by 3 Å heavy atom RMSD) showed that opposite binding modes, where HMF is flipped in the active site so that the opposite functional group is closer to the catalytic centre, are also plausible, thus, the binding energies of clusters suggesting opposite selectivity are very close ($\Delta\Delta G_{\text{Binding}} < 1$ kcal/mol). This may be explained by the notably hydrophobic nature of UPOs' active site, exemplified by *HspUPO* in Figure 3. In fact, no possible polar contacts between HMF and the UPO pocket were observed in any of the docking modes. Thus, the experimentally observed excellent chemoselectivity of *ApsUPO* (molar ratio DFF:HMFCFA, 99:1) and *MorUPO* (molar ratio DFF:HMFCFA, 95:5) for the alcohol oxidation in the first step cannot be adequately explained through molecular docking studies.

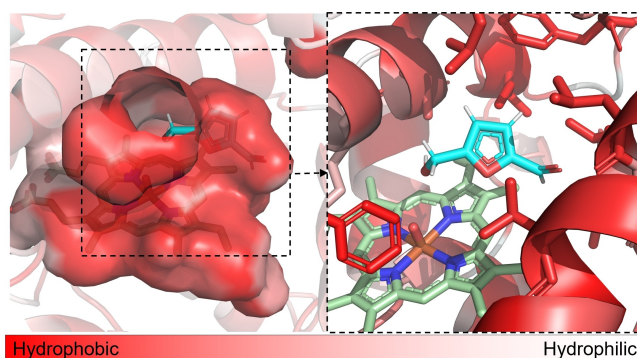


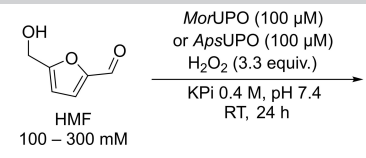
Figure 3. Active site hydrophobicity of UPOs exemplified by *HspUPO*. Hydrophobicity scale as defined by 'normalized consensus hydrophobicity scale'.^[23] Hydrophobicity color gradient: red indicates areas of high hydrophobicity, white indicates hydrophilic areas. The heme cofactor (Cpd-I) is shown in pale green and HMF in cyan.

Cascades with *ApsUPO* and *MorUPO*

Since the chemoselective of *HspUPO* in the first oxidation step led always to significant quantities of HMFCFA, we turned our attention to the two other most active UPOs from our initial screening, namely *ApsUPO* and *MorUPO*. Considering, that HMFCFA is almost a dead-end product, the chemoselectivity for the 'alcohol 1st pathway' in the initial screening at 10 mM substrate concentration was found to be higher for these enzymes than for *HspUPO* [*ApsUPO*: 99:1 (alcohol 1st):(aldehyde 1st); *MorUPO*; 93:7; *HspUPO*: 74:26]. Subsequently, these two UPOs were investigated for the oxidation of HMF at elevated substrate concentrations (Table 2).

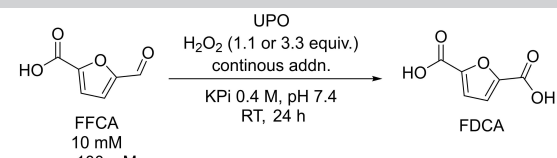
Remarkably, at 100 mM of HMF *MorUPO* converted 99% of the substrate with 95% selectivity for the 'alcohol 1st pathway' giving 83% of FFCA as well as 11% of the dicarboxylic acid product FDCA. Although *ApsUPO* displayed even higher chemoselectivity in the first oxidation step, *MorUPO* performed significantly better at 100 mM substrate concentration compared to *ApsUPO* which achieved only 38% conversion of HMF. Increasing the substrate concentration further to 200 or 300 mM, while keeping the *MorUPO* concentration constant, led to increased turnover numbers for the UPO but also to incomplete conversion of the substrate. The FFCA concentration (83 mM) obtained here by a single biocatalyst (*MorUPO*) at 100 mM substrate concentration has only been outperformed in a literature report requiring a three enzyme system comprising the galactose oxidase from *Dactylium dendroides* (*DdGO*), the alcohol dehydrogenase from *Synechocystis sp.* (*SynADH*) and horseradish peroxidase (HRP) converting 100 mM HMF to FFCA (97% conv.).^[24]

With a robust and chemoselective biocatalyst to access FFCA in hand, we tested *HspUPO*, *ApsUPO*, *MorUPO* and *AaeUPO* as potential candidates to perform the final oxidation step, thus, the oxidation of FFCA to FDCA (Table 3). Since both *HspUPO* and *AaeUPO* reached completion in the oxidation at 10 mM of FFCA as the substrate, these two UPOs were tested at 100 mM FFCA concentration also varying the H₂O₂ concentration. *AaeUPO* turned out to be superior to *HspUPO* reaching

Table 2. Testing chemoselective *ApsUPO* and *MorUPO* at elevated HMF concentrations.


UPO	HMF Conc. [mM]	Molar product ratio [%]					TON
		HMF	DFF	HMFCa	FFCA	FDCA	
<i>ApsUPO</i>	100	62	30	0	8	0	460
<i>MorUPO</i>	100	1	0	5	83	11	1872
<i>MorUPO</i>	200	22	0	5	64	9	3262
<i>MorUPO</i>	300	35	0	5	50	10	3828

^[a] Conditions: HMF (100 – 300 mM), UPO (100 μM), H₂O₂ (330 – 990 mM, 13.75 – 41.25 mM h⁻¹) KPi 0.4 M, pH 7.4, room temperature, 24 h. Conversion & molar product ratio were determined on HPLC by external calibration with commercial reference material.

Table 3. Oxidation of FFCA by selected UPOs, varying the substrate and H₂O₂ concentration.


UPO	FFCA [mM]	H ₂ O ₂ (equiv.)	conv. [%]
<i>HspUPO</i>	10	3.3	> 99
<i>ApsUPO</i>	10	3.3	4
<i>MorUPO</i>	10	3.3	23
<i>AaeUPO</i>	10	3.3	> 99
Control (no UPO)	10	3.3	2
<i>HspUPO</i>	100	1.1	35
<i>HspUPO</i>	100	3.3	42
<i>AaeUPO</i>	100	1.1	40
<i>AaeUPO</i>	100	3.3	91
Control (no UPO)	100	1.1	6
Control (no UPO)	100	3.3	17

^[a] Conditions: FFCA (10 or 100 mM), UPO (10 μM for 10 mM Substrate, 25 μM for 100 mM Substrate), H₂O₂ (33 mM - 330 mM, 1.375 to 13.75 mM h⁻¹) KPi 0.4 M, pH 7.4, room temperature, 24 h. Conversion & molar product ratio were determined on HPLC by external calibration with commercial reference material.

91 % conversion to FDCA with 3.3. equivalents of H₂O₂, which is in line with the pronounced catalase activity reported for this UPO.^[25]

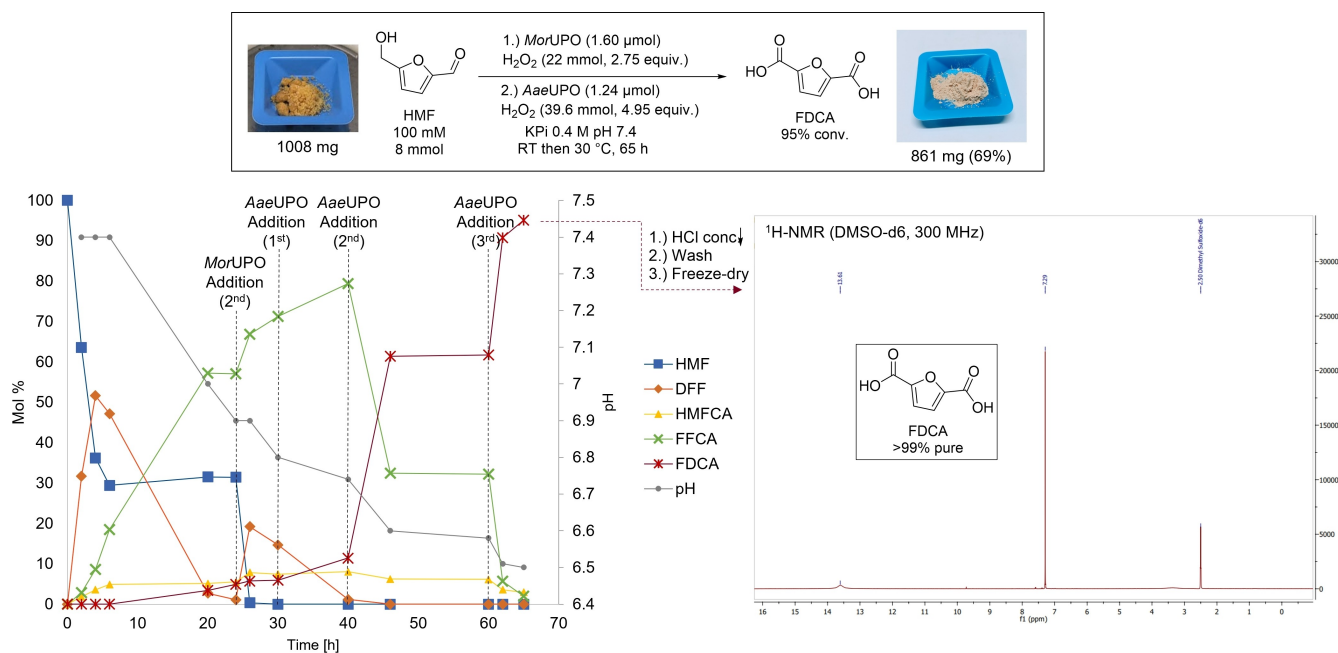
As observed in the control reaction without UPO, FFCA is also oxidized to FDCA with H₂O₂ in the absence of an enzyme. This suggests that that a fraction of the FDCA generated in the HMF oxidation by *MorUPO* (Table 2) might stem from the spontaneous H₂O₂-induced background oxidation.

Two UPOs in a One-Pot Cascade for the Synthesis of FDCA

Since *AaeUPO* was identified as suitable catalyst for FFCA oxidation but was not chemoselective for the oxidation of HMF

as reported before^[12] and confirmed in our initial screening (Table 1) and also inefficient in oxidizing HMFCa, a one-pot, two-step process was set up, using *MorUPO* for the chemoselective oxidation of HMF to give FFCA and then adding *AaeUPO* for the final oxidation step (Scheme 2).

To isolate the product, we transferred the analytical scale conditions (Table 2 & Table 3) to a semi-preparative scale (38 mg) (Table 4). By this approach the dicarboxylic acid FDCA was obtained with > 99% conv. as the sole product. Notably, *AaeUPO* converted also the small amount of HMFCa formed to FDCA. This allowed to obtain FDCA in pure form with 78% isolated yield (35 mg) as confirmed by HPLC and also NMR analysis after acidic workup and freeze drying. A pure FDCA product is crucial for the use as a polymer precursor since e.g.



Scheme 2. Gram scale synthesis of FDCA with a biocatalytic one-pot cascade employing two UPOs. Molar percentages of oxidation products (mol %) were determined on HPLC by external calibration with commercial reference material. Lines between data points are just a guide for the eye and do not represent actual data.

Table 4. UPOs employed in the synthesis of FDCA.

Catalysts	HMF [mM]	Conv. [%]	Isolated Yield [%]	TON (for UPO)	Productivity ^[f] (FDCA) [mg L ⁻¹ h ⁻¹]
<i>AaeUPO</i> , <i>PeAAO</i> ^[12]	3	91	n.d.	<i>AaeUPO</i> : 4154	36
<i>AaeUPO</i> , <i>PeAAO</i> , <i>DdGO</i> ^[13]	10	80	n.d.	<i>AaeUPO</i> : 444 ^[b]	52
<i>AaeUPO</i> , <i>PeAAO</i> , <i>PpAO</i> ^[11]	1.5 ^[a]	98	n.d.	<i>AaeUPO</i> : 594	2
<i>MorUPO</i> , <i>AaeUPO</i> , 38 mg scale (<i>this work</i>)	100	> 99	35 mg (78)	<i>MorUPO</i> :1872 (3828) ^[c] <i>AaeUPO</i> : 1860 (3640) ^[d]	215
<i>MorUPO</i> , <i>AaeUPO</i> , 1 g scale (<i>this work</i>)	100	95	861 mg (69)	<i>MorUPO</i> : 9131 (13535) ^[e] <i>AaeUPO</i> : 7079	122

^[a] 5-(Methoxymethyl)furfural (MMF) was used as the substrate.^[b] FFCA as the substrate in an isolated reaction using only *AaeUPO* and H₂O₂ (TON calculated, assuming reported UPO concentration of 0.04 mg mL⁻¹ equals 0.9 μM). ^[c] Higher TONs were achieved for *MorUPO* with increased substrate concentration (see Table 2). ^[d] Higher TONs were achieved for *AaeUPO* with FFCA (100 mM) as the substrate (Table 3). ^[e] TON before 2nd addition of *MorUPO*. ^[f] Productivity is based on conversion for comparability. *PeAAO* = aryl alcohol oxidase from *Pleurotus eryngii*, *PpAO* = alcohol oxidase from *Pichia pastoris*.^[28] *DdGO* = galactose oxidase from *Dactylium dendroides*.^[29] n.d. not determined (product was not isolated).

HMFCFA would influence the chain growth during polymerization.^[26] To investigate the cascade on even larger scale, it was repeated on gram scale, reducing the concentration of enzyme with respect to the analytical scale conditions (from 100 μM to 10 μM starting concentration for *MorUPO*, from 25 μM to 3 μM for *AaeUPO*, see experimental section for details) (Scheme 2). The idea being to monitor the reaction and add further enzyme if needed. Also, the concentration of the H₂O₂ solution continuously added was reduced from 3.3 M to 0.88 M to further minimize the oxidative burden for the UPOs. To our delight, *MorUPO* worked efficiently under these conditions exhibiting a TON of 13535 in the first step at 24 h. An equal amount of *MorUPO* was added and the oxidation of HMF was

completed after additional six hours (t=30 h) leading to an overall TON of 9131 for the first step with *MorUPO*. To complete the oxidation towards the desired FDCA, *AaeUPO* was added in total three times at t=30 h, 40 h and 60 h, achieving 95% conversion and an overall TON of 7079 (see experimental section for details). Due to the mediocre performance of *AaeUPO* upon its first addition the temperature was raised from room temperature (22 °C) to 30 °C (t=40 h until t=65 h).

As highlighted by multiple researchers and in a dedicated study,^[27] FDCA exhibits limited solubility in common employed solvents. The FDCA concentration achieved here in aqueous buffer as well as previously reported in biocatalytic literature^[7] far exceeded the documented solubility in water (11.3 mM in

H₂O at 40 °C),^[27] thus, no precipitation was observed, which is advantageous for preparative transformations.^[14] The enhanced solubility is likely connected to the buffered system used here compared to the solubility reported in pure H₂O.

Comparing the here presented one pot cascade with literature reporting UPOs for the synthesis of FDCA (Table 4), one can find three studies in which always *AaeUPO* was used in combination with aryl alcohol oxidase from *Pleurotus eryngii* (*PeAAO*) and either galactose oxidase from *Dactylium dendroides* (*DdGO*) or alcohol oxidase from *Pichia pastoris*.^[11–13] Thereby *AaeUPO* was in general applied for the final oxidation step from FFCA to FDCA, with TONs reaching from 444 to 4154 (Table 4). Thus, the here reported TONs surpass literature in the experiment on gram scale and also the substrate concentration was here increased at least 10-fold. Thus, the study here represents the first example to use exclusively UPOs to reach FDCA at 100 mM substrate concentration allowing also to get the isolated product in pure form.

Conclusions

This study showed that the two UPOs *MorUPO* and *ApsUPO* can be used as chemoselective biocatalysts for the synthesis of FFCA starting from HMF at 100 mM. Probably even more important, this study demonstrates the successful synthesis of FDCA from HMF via a three step one pot oxidative cascade using UPOs only (*MorUPO* and *AaeUPO*). The approach allowed to start from an elevated substrate concentration of 100 mM and gram scale, surpassing previous reports performed on analytical scale and getting FDCA with 95–99% conv. and 69–78% isolated yield. These results demonstrate the potential for the scalable synthesis of FDCA by UPOs, considering that wild-type UPOs were utilized in this study, the process most likely can be improved even further.

The utilization of biocatalysis in the oxidation of HMF to FDCA not only offers selectivity advantages over traditional chemical methods but also aligns with the principles of green chemistry by minimizing waste as only H₂O₂ is required as reagent giving finally just water as side product and reducing energy consumption as the reaction can be performed at ambient temperature.

Experimental Section

General Procedure for Biotransformations with UPOs on Analytical Scale

Procedure: All reactions were performed in duplicates in 1.5 mL glass vials with 1 mL of total volume. The enzyme (resuspended lyophilized enzyme preparation, 100 μ L, 10–100 μ M) was added to a solution of the substrate (800 μ L, 12.5 mM) in KPi buffer (100 mM pH 7.4). For biotransformations with higher substrate or enzyme concentrations the concentration of the stock solution was adjusted accordingly, and the buffer strength was increased (400 mM pH 7.4). To start the reaction a H₂O₂ solution (100 μ L, 330 mM in reaction buffer, 3.3 equiv.) was continuously added over 24 or 48 hours with a kdScientific pump, equipped with 1 mL Omnifix®-F

syringes from BRAUN (\varnothing 4.7 mm) and 100 Sterican® needles (\varnothing 0.80 mm x 120 mm). **Workup:** An aliquot of 50 μ L was taken and 1 μ L of catalase (from bovine liver, 4 mg/mL) was added to quench residual H₂O₂. Then, 50 μ L of diluted H₂SO₄ (0.5 M) was added, followed by the addition of 200 μ L MeCN with 2-furoic acid as internal standard (1 mM) (final dilution factor=6). For reactions with higher substrate concentration (50 mM and above) the sample was further diluted by adding 900 μ L of a solution (1:1:4 Buffer: H₂SO₄:MeCN/2-Furoic acid [1 mM]) (dilution factor=24). The mixture was vortexed and centrifuged, and the supernatant was subjected to analysis. **Analytcs:** Conversion & molar product ratio were determined on HPLC by external calibration with commercial reference material at 280 nm (HMF, DFF, FFCA) or 254 nm (HMFCFA, FDCA). Column: Phenomenex Rezex Organic Acid H⁺ (8%), 300 x 7.8 mm; Eluent: H₂SO₄ 2.5 mM pH=2.6; Flow=0.8 mL/min; T=60 °C (Operating pressure=29 bar). Retention times: FDCA 10.32 min, FFCA 13.62 min, HMFCFA 14.58 min, HMF 22.59 min, DFF 27.14 min.

Gram Scale One-Pot Cascade for the Synthesis of FDCA

Procedure: Step 1.) with *MorUPO*: HMF (1008 mg, 8 mmol) was added to a 250 mL round bottom flask and dissolved in KPi (75 mL, 0.4 M pH 7.4). A lyophilized *MorUPO* preparation (89 mg, 0.8 μ mol) was resuspended in KPi (5 mL, 0.4 M, pH 7.4) and added to the flask. The reaction was started by adding a diluted H₂O₂ solution (20 mL, 880 mM in dH₂O, 0.833 mL h⁻¹ for 24 h, 17.6 mmol, 2.2 equiv.) continuously with a kdScientific pump, equipped with a 20 mL Omnifix®-F syringes from BRAUN (\varnothing 20.1 mm) attached to a Teflon tube. After completion of H₂O₂ addition bovine liver catalase was added (0.1 mg ~1000 U) and stirred for 10 min at RT (22 °C) to quench residual H₂O₂. The reaction was heated to 85 °C for 10 min to deactivate the catalase, then cooled to RT. *MorUPO* (89 mg, 0.8 μ mol) resuspended in KPi (5 mL, 0.4 M, pH 7.4) and H₂O₂ (5 mL, 880 mM in dH₂O, 0.833 mL h⁻¹ for 6 h, 4.4 mmol, 0.55 equiv.) were added again to complete oxidation of HMF. Step 2.) with *AaeUPO*: A lyophilized *AaeUPO* preparation (112.5 mg, 0.31 μ mol) was resuspended in KPi (3 mL, 0.4 M, pH 7.4) and added to the flask followed by the addition of H₂O₂ (20 mL, 880 mM in dH₂O, 2 mL h⁻¹ for 10 h, 17.6 mmol, 2.2 equiv.). After completion of H₂O₂ addition bovine liver catalase was added (0.1 mg ~1000 U) and stirred for 10 min at RT (22 °C) to quench residual H₂O₂. The reaction was heated to 85 °C for 10 min to deactivate the catalase, then cooled to RT. *AaeUPO* (112.5 mg, 0.31 μ mol) resuspended in KPi (3 mL, 0.4 M, pH 7.4) and H₂O₂ (20 mL, 880 mM in dH₂O, 1 mL h⁻¹ for 20 h, 17.6 mmol, 2.2 equiv.) were added again. Another catalase/heat/cool cycle was performed as described above before the last addition of *AaeUPO* (225 mg, 0.62 μ mol) and H₂O₂ (5 mL, 880 mM in dH₂O, 1 mL min⁻¹ for 5 h, 4.4 mmol, 0.55 equiv.). To monitor the reaction, aliquots (50 μ L) were withdrawn from the cascade and worked up as described in the general procedure above. **Workup** is based on an adapted protocol from literature.^[7] The reaction mixture was heated to 85 °C for 10 min, cooled to room temperature and centrifuged to remove the denatured biocatalyst. The supernatant was cooled to 0 °C and FDCA was precipitated by adding conc. HCl dropwise until pH 1.5. The mixture was centrifuged, and the precipitate was washed with 1 M HCl. Here it should be mentioned that washing with aqueous 1 M HCl is crucial since washing with, e.g., dH₂O only leads to a poorly soluble solid, an insight that partially reflects the mediocre isolated yield obtained. The crude product was dried under reduced pressure to remove any residual HCl and final drying was performed by lyophilization. **Analytcs:** The dry, pure product (861 mg, 69% yield) was analysed by NMR. ¹H NMR (DMSO-d₆, 300 MHz): 7.29 (s, 2 H, Ar-H), 13.61 (br. s, 2 H, COO-H). Spectroscopic data agrees with literature.^[30]

Computational Methods

Docking experiments were performed with the YASARA Structure software^[31] and the implemented AutoDock Vina algorithm.^[32] The AMBER force field^[33] was used and the ligands were energy minimized with the AMBER^[34] force field before docking. The simulation cell was defined at 10 Å around the iron atom of the heme and 100 docking trials were run. The results were visualized via the PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC. Homology models of UPOs were generated using ColabFold v1.5.2-patch: AlphaFold2^[35] using MMseqs2.^[36] The heme cofactor was docked into the active site of the UPO model as described before^[20] and the ferryl oxygen of Cpd-I was modelled 1.64 Å from the iron centre.^[37]

Supporting Information

The authors have cited additional references within the Supporting Information.^[38]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Unspecific Peroxygenases · Biocatalysis · 2,5-Furandicarboxylic acid · 5-(Hydroxymethyl)furfural · Sustainable Chemistry

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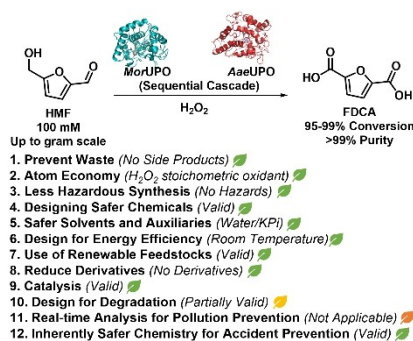
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RESEARCH ARTICLE

A scalable one-pot cascade for the oxidation of 5-(hydroxymethyl)furfural (HMF) and subsequent isolation of 2,5-furandicarboxylic acid (FDCA) was successfully performed using two un-specific peroxygenases (UPOs). The UPO from *Marasmius oreades* (*MorUPO*) oxidized HMF to 5-formyl-2-furoic acid (FFCA) with 95% chemoselectivity. In a sequential step, the UPO from *Agrocybe aegerita* (*AaeUPO*) completed the oxidation-cascade, achieving up to 99% conversion to FDCA and yielding 861 mg isolated pure crystalline FDCA.



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Multistep Biooxidation of 5-(Hydroxymethyl)furfural to 2,5-Furandicarboxylic Acid with H_2O_2 by Un-specific Peroxygenases

