Self-sustained enzymatic cascade for the production of 2,5-furandicarboxylic acid from 5-methoxymethylfurfural

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Abstract

**Background**: 2,5-Furandicarboxylic acid is a renewable building block for the production of polyfurandicarboxylates, which are biodegradable polyesters expected to substitute their classical counterparts derived from fossil resources. It may be produced from bio-based 5-hydroxymethylfurfural or 5-methoxymethylfurfural, both obtained by the acidic dehydration of biomass-derived fructose. 5-Methoxymethylfurfural, which is produced in the presence of methanol, generates less by-products and exhibits better storage stability than 5-hydroxymethylfurfural being, therefore, the industrial substrate of choice.

**Results**: In this work, an enzymatic cascade involving three fungal oxidoreductases has been developed for the production of 2,5-furandicarboxylic acid from 5-methoxymethylfurfural. Aryl-alcohol oxidase and unspecific peroxygenase act on 5-methoxymethylfurfural and its partially oxidized derivatives yielding 2,5-furandicarboxylic acid, as well as methanol as a by-product. Methanol oxidase takes advantage of the methanol released for in situ producing H₂O₂ that, along with that produced by aryl-alcohol oxidase, fuels the peroxygenase reactions. In this way, the enzymatic cascade proceeds independently, with the only input of atmospheric O₂ to attain a 70% conversion of initial 5-methoxymethylfurfural. The addition of some exogenous methanol to the reaction further improves the yield to attain an almost complete conversion of 5-methoxymethylfurfural into 2,5-furandicarboxylic acid.

**Conclusions**: The synergistic action of aryl-alcohol oxidase and unspecific peroxygenase in the presence of 5-methoxymethylfurfural and O₂ is sufficient for the production of 2,5-furandicarboxylic acid. The addition of methanol oxidase to the enzymatic cascade increases the 2,5-furandicarboxylic acid yields by oxidizing a reaction by-product to fuel the peroxygenase reactions.

**Keywords**: 2,5-Furandicarboxylic acid, 5-Methoxymethyl furfural, Enzyme cascade, Biocatalysis, Oxidase, Peroxygenase, Renewable polyesters

Background

Fossil resources are finite and the need for substituting petroleum-based materials with renewable materials is increasing in recent years [1]. 2,5-Furandicarboxylic acid (FDCA) is nowadays regarded as a promising precursor for the production of renewable and biodegradable bioplastics. Polyester formed by the condensation of this building block with ethylene glycol, known as poly(ethylene-2,5-furandicarboxylate) (PEF), is expected to substitute for other polyesters produced from fossil fuels, thanks to their renewable origin and their mechanical and gas barrier properties, which are even better than those of conventional poly(ethylene terephthalate) (PET) [2, 3]. Therefore, it is expected that PEF will be able to compete with PET not only in economic but also in environmental terms since its production lowers the balance of greenhouse gases emissions.

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The first report on PEF enzymatic hydrolysis, which permits the recycling of its monomers, has been brought to light recently [5].

FDCA can be obtained from precursors that are formed upon the acidic dehydration of fructose, directly obtained from plants (as monosaccharide, in sucrose disaccharide and in inulin-type polymers) or by isomerization of glucose from hydrolysis of disaccharides (e.g., sucrose) or polysaccharides (e.g., in lignocellulosic materials). These precursors are mainly 5-hydroxymethylfurfural (HMF) and more recently 5-methoxymethylfurfural (MMF). The latter is obtained when fructose is dehydrated in the presence of methanol or by HMF etherification [6–8]. MMF is more stable upon storage than HMF, and fructose dehydration in methanol yields less side-products than when it takes place in water for HMF production. Successful attempts have been made to obtain polyesters from MMF and its derivatives [9], and a joint venture between BASF and Avantium, Synvina (www.synvina.com), has been created for sustainable industrial production of PEF from stable MMF.

In the above context, several patents [10–12] present methods for the production of FDCA from MMF, but all of them use oxidation catalysts such as bromide, cobalt, or manganese, along with other metals. Moreover, they describe processes that take place at high temperatures (in the range of 100–220 °C) and pressures (3–15 bar).

The advantage of enzymes, which work under mild conditions (in aqueous solution, at room temperature and under atmospheric pressure), for the production of FDCA has gained momentum and several reports on the enzymatic oxidation of HMF to FDCA are available [13–15]. Particularly, the use of the natural portfolio of oxidases and peroxygenases in synthetic chemistry is very timely. While the former can perform selective oxidations producing H₂O₂ from atmospheric O₂, the latter can use the released H₂O₂ to complete the full oxidation of complex molecules like in the whole conversion of HMF to FDCA which comprises three sequential oxidation steps [13].

MMF conversion into FDCA can involve three or four oxidation steps depending on whether the ether breakdown leaves an alcohol or a carbonyl function in the furfural molecule (Fig. 1 scheme, pathways 1/3–5 or 1/2/5, respectively). In the present study, a new self-sustained enzymatic cascade was developed for the production of FDCA from MMF—combining aryl-alcohol oxidase (AAO) [16], unspecific peroxygenase (UPO) [17], and methanol oxidase (MOX) [18]—identified the intermediate products by gas chromatography-mass spectrometry (GC–MS), estimated the conversion yields and established the oxidation pathway.

**Results**

**Hydration of the carboxyl group in MMF**

Comparison of ¹H-NMR spectra in deuterated water and deuterated dimethylsulfoxide (DMSO-d₆) allows detection of the aldehyde and the geminal diol signals to measure the degree of hydration at equilibrium. The MMF spectrum in DMSO-d₆ showed six signals assigned to the aldehyde (9.6 ppm), the furanic ring (7.5 and 6.7 ppm), methylene ether (4.5 ppm), methyl (3.3 ppm), and residual DMSO (2.5 ppm) protons. On the contrary, the spectrum in sodium phosphate (pD 7.0) gave 8 signals assigned to the aldehyde proton (9.6 ppm) and its shifted counterpart hemiacetalic hydrated form (small signal at 6.8 ppm), the two ring protons (7.7 and 6.9 ppm) and their shifted counterparts (small signal at 6.5 ppm), the methylene ether (4.5 ppm) methyl (3.3 ppm), and residual DMSO (2.5 ppm) protons. On the contrary, the spectrum in sodium phosphate (pD 7.0) gave 8 signals assigned to the aldehyde proton (9.6 ppm) and its shifted counterpart hemiacetalic hydrated form (small signal at 6.8 ppm), the two ring protons (7.7 and 6.9 ppm) and their shifted counterparts (small signal at 6.5 ppm), the methylene ether (4.7 ppm) and methyl (3.5 ppm) protons, as well as the water protons (4.9 ppm). Integration of the aldehyde signal and its small shifted counterpart points towards a degree of MMF hydration ≤ 10%.

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![Fig. 1](image-url)  
Fig. 1. Scheme of the possible pathways for the oxidation of MMF into FDCA. MMF, 5-methoxymethylfurfural; MMFA, 5-methoxymethylfuran carboxylic acid; HMFCA, 5-hydroxymethyl-2-furan carboxylic acid; FFCA, 5-formylfuran carboxylic acid; and FDCA, 2,5-furandicarboxylic acid.
MMF oxidation by AAO

AAO may oxidize aldehydes to acids if their carbonyl groups are partially hydrated to gem-diols [19]. To test the ability of AAO from Pleurotus eryngii to oxidize the gem-diol form of MMF, the compound was incubated with the enzyme and the reaction was analyzed by GC–MS. The reaction was completed after 15 h, using a substrate/enzyme ratio of 300 under the conditions described above (Fig. 2a). 5-(Methoxymethyl)-2-furancarboxylic acid (MMFA) accumulated over time and additional products were not detected, confirming that AAO does not show any activity on the resulting molecule. The above results show that the small hydration degree shown by NMR was enough for AAO oxidation of the MMF molecule to MMFA (step 1 in Fig. 1 scheme).

The kinetic constants for the AAO oxidation of MMF and related furfurals were indirectly measured as H₂O₂ release, by coupling the reaction of horseradish peroxidase (HRP) and a reagent that gives a colored product when peroxide is available (Table 1). Comparison with the kinetic constants estimated for related furfurals shows that the methoxyl moiety in MMF decreases the enzyme affinity ($K_m$ is increased) and lowers reactivity ($k_{cat}$ is slightly reduced). Both effects together result in much lower AAO catalytic efficiency for MMF than for the other two furfurals assayed.

Residual activity estimations showed that AAO was active during the whole time of the reaction (dashed line in Fig. 2a) displaying a half-life of around 6 h that guaranteed the full conversion of MMF into MMFA. Catalytic performance parameters of AAO, including residual activity together with turnover number (TON), turnover frequency (TOF), and total turnover number (TTN) values under the described conditions, are provided in Table 2.

![Fig. 2](image-url) Time course of the reaction of MMF (1.5 mM) with: a AAO, b AAO, UPO and H₂O₂, c AAO, UPO and MOX, and d AAO, UPO, MOX and methanol (at 28 °C in 100 mM phosphate, pH 7). Enzyme concentrations were 5 µM (AAO and UPO) and 1 µM (MOX), while H₂O₂ and methanol final concentrations (in b and d, respectively) were 1.5 mM (added after 24, 48 and 72 h) and 1 mM (added after 72 and 96 h reaction). Dashed lines in a and b represent the AAO and UPO residual activities as a function of time, respectively. Compounds were identified and quantified by GC–MS, using the estimated response factors.
UPO reactions and AAO/UPO cascade

For the desired reaction to proceed further (from MMFA to FDCA), it was necessary to find a catalyst that could cleave the ether bond of the methoxyl group, to hydroxylate the 5-formylfurancarboxylic acid (FFCA) molecule and, if the ether cleavage left a hydroxyl group in the molecule, to oxidize the 5-hydroxymethyl-2-furancarboxylic acid (HMFCA) molecule to FFCA (steps 2/5 or 3–5, respectively, in Fig. 1 scheme). In this regard, the UPO from Agrocybe aegerita has been reported to cleave a variety of ether bonds [20], as well as to hydroxylate FFCA to FDCA in the presence of H2O2 [13].

To clarify the enzymatic pathway, UPO (5 µM) was incubated with MMFA (1.5 mM) in the presence of H2O2 (1.5 mM, final concentration). Detection of FFCA as the sole product (data not shown) revealed that UPO is indeed capable to cleave the ether bond of MMFA while forming an additional carbonyl group in the molecule. This suggests that the reaction mainly proceeds through step 2 (Fig. 1 scheme), rather than through steps 3 and 4, although traces of HMFCA were detected as well. Therefore, only steps 1, 2, and 5 in the Fig. 1 scheme would be required for the production of FDCA from MMF, which is advantageous, since it saves one catalytic step that would require additional H2O2.

Addition of exogenous H2O2 to the cascade

To test if the limited FDCA yields of the AAO/UPO cascade were due to the depletion of H2O2, this stoichiometric UPO substrate was added at different times after 24 h of reaction (up to 1.5 mM) and the products were analyzed during the subsequent incubation. Analysis of the reaction products showed that the whole reaction was improved upon the addition of H2O2. The FDCA yield corresponded to 40% conversion of initial MMF and the remaining 60% accounted for MMFA, while only traces of FFCA were detected (Figs. 2b and 3). Although the concentration of H2O2 (exogenously added and in situ produced by AAO) may have been sufficient, the complete conversion of MMF into FDCA was not achieved.

UPO showed activity during the whole process, displaying robustness as a biocatalyst by maintaining high levels of residual activity throughout the reaction (Fig. 2b, dashed line). UPO half-life and other catalytic performance parameters (TON, TOF, and TTN) under

### Table 1 Catalytic constants for the oxidation of different furfurals by AAO

|       | k_cat (min⁻¹) | k_m (mM) | k_cat/k_m (min⁻¹ mM⁻¹) |
|-------|---------------|----------|-----------------------|
| MMF   | 15.8 ± 0.6    | 60.8 ± 5.5 | 0.35 ± 0.02           |
| HMF   | 20.1 ± 0.6    | 1.6 ± 0.2  | 12.9 ± 1.2            |
| DFF   | 31.4 ± 0.7    | 3.3 ± 0.2  | 9.4 ± 0.5             |

Reactions measured in 50 mM sodium phosphate (pH 7.0) at 25 °C. Means and standard deviations estimated from the fit to Michaelis–Menten equation. Kinetics were measured by triplicates.

### Table 2 Other catalytic parameters of AAO and UPO reactions (in Fig. 1a and b)

|       | Half-life (h) | TTN | TON | TOF (h⁻¹) |
|-------|---------------|-----|-----|-----------|
| AAO   | 6.3           | 8620| 300 | 20        |
| UPO   | 112.0         | 594 | 594 | 5         |

TTN, TON and TOF were calculated using Eqs. 3–5, respectively (reaction times were 15 h for AAO and 120 h for UPO). Parameters estimated from single reactions using MMF (1.5 mM) as substrate and AAO and UPO (5 µM) as biocatalysts at pH 7.0 and 28 °C.
the assayed conditions are shown in Table 2, together with those of AAO.

Improvement of FDCA yield by MOX (and methanol) addition

The peroxygenase activity of UPO enables it to insert one O atom, which leads to ether breakdown, concomitantly with the formation of a carbonyl group in one of the products and a hydroxyl group in the other product [20]. As described above, the product of the reaction of MMFA with UPO was FFCA, in which a new carbonyl group was introduced. Consequently, UPO would release methanol as second fission product of the ‘quasi-benzylic’ peroxygenation reaction.

Therefore, with the aim of producing additional H₂O₂ to fuel the UPO reactions, commercially available MOX from Pichia pastoris was added to the enzymatic cascade described above to a final concentration of 1 µM (substrate/enzyme ratio of 1500). In this case, MOX catalyzes its canonical reaction, the oxidation of methanol to methanal and concomitantly, the reduction of O₂ to H₂O₂. GC–MS analysis showed that upon the addition of MOX and the resulting increase of available H₂O₂, the UPO conversion was enhanced and FDCA yield reached 70% of the initial MMF concentration (Figs. 2c and 3). According to the analysis of products, the rate-limiting step of the UPO reactions (step 2 in Fig. 1 scheme) was demethoxylation, since MMFA was always the most abundant intermediate (up to 80% of initial MMF, after 24 h) compared to smaller amounts of FFCA (around 10% of the applied MMF).

To determine whether the improved FDCA yield (70%) was still limited by the amount of H₂O₂ available, exogenous methanol (1 mM final concentration) was added to the reaction after 72 and 96 h. In fact, with the addition of methanol (and subsequent H₂O₂ production), the FDCA formation further increased (Figs. 2d and 3), suggesting that the reaction was not limited by the activity of the biocatalyst. Thus, the FDCA conversion rose to 98% after 120 h, indicating that the limiting factor of the whole enzymatic cascade was the H₂O₂ availability. A summary of the conversion yields of MMF into MMFA, FFCA, and FDCA during operation (0–120 h) of the methanol-supplemented AAO/UPO/MOX cascade is provided in Table 3.

**Table 3 Summary of the MMF (1.5 mM) conversion rates (mole %) to its three oxidized derivatives in the AAO/UPO/MOX cascade, supplemented with methanol (1 mM), at different reaction times**

| Time (h) | MMF | MMFA | FFCA | FDCA |
|---------|-----|------|------|------|
| 0       | 100 | 0    | 0    | 0    |
| 25      | 0   | 66   | 10   | 24   |
| 49      | 0   | 46   | 9    | 45   |
| 73      | 0   | 35   | 6    | 59   |
| 120     | 0   | 2    | 0    | 98   |

Compounds were identified and quantified by GC–MS, using the estimated response factors.
need for H$_2$O$_2$ input. In general, the oxidation of MMF by AAO and UPO seems to proceed more efficiently than that of HMF [13, 28], due to the different polarity/reactivity of the methyl-ether functionality and the primary alcohol group, respectively.

Although the catalytic performances of the two biocatalysts are modest, with TTN values lower than 10$^4$ and $k_{cat}$ values lower than 1 s$^{-1}$, the AAO/UPO cascade represents a good starting point for further improvement. The optimization of substrate and enzyme concentrations, required for an industrial exploitation of the cascade, would result in higher catalytic performance parameters.

The fact that UPO must catalyze two reactions (steps 2 and 5 in Fig. 1 scheme), whereas AAO does only one (step 1), causes a shortage in H$_2$O$_2$, so that the desired reactions cannot be completed (Fig. 3, blue dotted line). To solve this limitation, a three-member enzymatic cascade was developed as discussed below.

**By-product oxidation to fuel the reaction**

The addition of a third biocatalyst, MOX demonstrated that the limited FDCA yield (< 40% in the two-member AAO/UPO cascade) can be overcome by the in situ production of additional H$_2$O$_2$ to be used by UPO. Thus, a conversion of 70% from MMF to FDCA was attained using a three-enzyme cascade (AAO/UPO/MOX), which is fueled by the reduction of H$_2$O$_2$ (by UPO) and the oxidation of methanol (by MOX), two by-products generated by AAO and UPO, respectively. The respective results indicate that the 70% conversion is attained solely by the agents involved in the reaction as long as there is an atmospheric O$_2$ input, as depicted in the three-member cascade scheme of Fig. 4.

The in situ self-generated fuel (H$_2$O$_2$ from the by-product methanol) here presented is a breakthrough in oxidative bioconversions. Other enzymatic cascades using UPO for oxygenations, in which methanol was applied as a sacrificial electron donor, have been reported, but the provenance of methanol was exogenous [26]. The production of methanal may be deleterious for enzymes if it reaches high concentrations, but such effect was not observed in the present study. The in situ generation of H$_2$O$_2$ has already been successfully applied in the bleaching industry using different flavoenzymes, such as glucose oxidase, alcohol oxidase [29] and cellobiose dehydrogenase/oxidase [30, 31].

Apart from improving the reaction yields, the cascade approach allows UPO to control the release of H$_2$O$_2$, since it is the enzyme itself that produces the methanol as substrate for MOX. It is well known that enzymes that bear heme groups, such as peroxidases and peroxygenases, are sensitive to high levels of their peroxide co-substrates resulting in deactivation via heme-bleaching.
In fact, comparing the FDCA yields of the three-catalyst cascade with the cascade involving AAO and UPO to which H₂O₂ was added clearly indicates that UPO reactions proceed better if H₂O₂ is gradually supplied in situ by enzyme action (see red vs orange lines in Fig. 3). The addition of exogenous H₂O₂ provokes a sharp rise of its concentration that may lead to UPO inactivation. This results in a decrease of the enzyme’s half-life, thus lowering TTN, which negatively affects the degree of conversion into FDCA. Although complete MMF conversion was not achieved using the AAO/UPO/MOX cascade, the addition of a small amount of ‘extra methanol’ to the reaction mixture resulted in almost complete conversion (98%) of MMF into FDCA (Fig. 3). In fact, an excess of H₂O₂ (above the stoichiometric quantity required for the peroxygenation reaction) was necessary to overcome the reported catalase side-activity of UPO [32], which may consume some of the H₂O₂ by producing H₂O and O₂.

**Conclusions**

In this work, a completely enzymatic approach to produce FDCA from MMF is reported for the first time. The synergistic activities of AAO and UPO catalyze such conversion through an enzymatic cascade involving the two biocatalysts and O₂ to trigger the reaction. UPO benefits from the H₂O₂ released by AAO to yield modest amounts of FDCA with H₂O and methanol as by-products. The addition of MOX, oxidizing methanol under release of additional H₂O₂, critically improved the FDCA yield that was before limited by the amount of H₂O₂ produced by AAO. Thus, 70% conversion was achieved with the only involvement of the three biocatalysts, O₂ and the by-products of the reaction, as illustrated in the Fig. 4 scheme. This yield could be further enhanced to 98% by the addition of some exogenous methanol resulting in additional H₂O₂ for UPO.

**Methods**

**Reagents**

*P. pastoris* MOX (EC 1.1.3.13), t-butyl-methyl-ether, MMFA, FDCA, N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and 2H₂O were purchased from Sigma-Aldrich (Saint Louis, MO, USA). MMF (=5-[methoxymethyl]-2-furancarboxaldehyde) was bought from AK Scientific, Inc (Union City, CA, USA). FFCA was purchased from TCI America (Portland, OR, USA). AmplexRed® and HRP were obtained from Invitrogen (Waltham, MA, USA). H₂O₂ and DMSO-d₆ were from Merck (Darmstadt, Hessen, Germany).

**Enzyme production**

AAO (EC 1.1.3.7) from the fungus *P. eryngii* was heterologously obtained from recombinant *Escherichia coli* W3110 harboring the pFLAG1 vector with the mature AAO cDNA (GenBank accession number AF064069). The enzyme was produced as inclusion bodies and further in vitro activated and purified as previously described [33].

PaDa-I variant of *A. aegerita* UPO (EC 1.11.2.1) was produced in *P. pastoris*, harboring the pPICZ-B-PaDa-I vector, grown in a 2-L glass fermentor. Expression was induced by the addition of methanol and the enzyme was chromatographically purified using Sepharose FF and Q-source columns (GE Healthcare, Piscataway, NJ, USA) as reported elsewhere [34, 35].

**Kinetic studies**

Kinetics of MMF, HMF, and DFF oxidation by AAO were studied by coupling the reaction of HRP and AmplexRed® at 25 °C, in 100 mM sodium phosphate, pH 7.0. H₂O₂ released by AAO is used by HRP to oxidize AmplexRed® to resorufin (Δε₅₆₃ = 52,000 M⁻¹ cm⁻¹) in a 1:1 stoichiometric fashion. Therefore, spectrophotometric monitoring of the formation of colored resorufin allowed the indirect measurement of the AAO kinetic constants. Increasing concentrations of MMF (8–250 mM) were mixed with AAO (0.5 µM), AmplexRed® (0.06 mM), and HRP (24 µg mL⁻¹) at a final volume of 1 mL. Reactions were triggered by addition of AAO and followed in a Cary 4000 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Kinetics were obtained from the linear phase of resorufin production as change in absorbance over time and averaged data for each substrate concentration were fitted to Michaelis–Menten equation to obtain the kinetic parameters using SigmaPlot software (Systat Software Inc., San Jose, CA, USA).

Residual activities of AAO and UPO were measured after different times of incubation in the presence of MMF and its oxidized derivatives. AAO residual activity was determined by following spectrophotometrically the production of p-anisaldehyde (Δε₂₈₅ = 16,950 M⁻¹ cm⁻¹) [36] from 200 µM *p*-methoxybenzyl alcohol, in 1 mL of 50 mM sodium phosphate, pH 6.0, at 25 °C. Regarding UPO, its residual activity was measured as the veratraldehyde (Δε₃₆₃ = 9300 M⁻¹ cm⁻¹) [32] produced from 10 mM veratryl alcohol and 2 mM H₂O₂, in 1 mL of 100 mM sodium phosphate, pH 7.0, at 25 °C. Experimentally determined values of residual activity were fitted to Eq. 1 describing the enzymatic activity loss as a function of time. This allowed estimation of the half-lives (Eq. 2) of AAO and UPO, as well as their TTN (Eq. 3, considering the enzyme half-life), TON (Eq. 4) and TOF (Eq. 5):

\[
\text{res. act.} = a \cdot e^{-\lambda \cdot t}
\]  \hspace{1cm} (1)

\[
t_{\frac{1}{2}} = \frac{\ln 2}{\lambda}
\]  \hspace{1cm} (2)
MMF oxidation reactions

MMF reactions were performed in 100 mM sodium phosphate (pH 7.0) under continuous shaking at 200 rpm in a thermostated chamber at 28 °C. In all of them, the substrate was added to a final concentration of 1.5 mM, while UPO and AAO final concentration was 5 µM. MOX attained a final concentration of 1 µM. All enzymes and substrates were added from the beginning of the reaction in the different reaction mixtures employed, except H₂O₂ (1.5 mM final concentration) and methanol (1 mM final concentration), which were gradually added after different incubation times (24, 48 and 72 h, and 72 and 96 h, respectively).

**GC–MS analyses**

250-µL samples were harvested from the one-pot reactions after different times to analyze the products present. Reactions were stopped by adding HCl to give pH 2–3. Low pH values cause protonation of the organic acids and permit their liquid–liquid extraction, which was carried out by mixing the reaction mixtures with an excess of t-butyl-methyl-ether three times, followed by treatment with anhydrous NaSO₄ to remove water traces. t-Butyl-methyl-ether was removed using a rotary evaporator at room temperature and samples were derivatized with 50 µL of BSTFA for 15 min at 25 °C [37].

Products were separated and identified using a gas chromatograph equipped with an HP-5MS column.

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**Fig. 5** Mass spectra of authentic standards of the compounds involved in the cascade, as trimethylsilyl (TMS) derivatives. a MMF b TMS-MMFA c TMS-FFCA d TMS-FDCA

\[
TTN = \frac{k_{\text{cat}} \cdot t_{1/2}}{\ln{2}} \tag{3}
\]

\[
TON = \frac{\text{mol product}}{\text{mol catalyst}} \tag{4}
\]

\[
TOF = \frac{TON}{\text{time}} \tag{5}
\]
(Agilent, Santa Clara, CA, USA; 30 m × 0.25 mm internal diameter; 0.25 μm film thickness) coupled to a quadrupole mass detector. The oven program started at 110 °C (maintained for 2 min), increasing at 20 °C·min⁻¹ until reaching 310 °C. Helium was used as the carrier gas at a flow rate of 1.2 mL·min⁻¹. The compounds involved in the MMF oxidative pathway were identified by comparing their mass spectra (and retention times) with those of derivatized authentic standards (Fig. 5). The following response factors were calculated as the slope of the fits of the responses of various concentrations of each standard compound (after its liquid–liquid extraction, derivatization and GC–MS analysis) to a linear equation: MMF: 1.7 × 10⁷ total-ion mM⁻¹; MMFA: 4.1 × 10⁷ total-ion mM⁻¹; FFCA: 3.1 × 10⁷ total-ion mM⁻¹; and FDCA: 3.6 × 10⁷ total-ion mM⁻¹. These response factors were used to estimate the mole percentage of each of the compounds in the reactions.

NMR studies

1H-NMR was used to investigate the degree of hydration of the carbonyl group in the MMF molecule, using a Bruker 500 MHz instrument (Billenica, MA, USA). MMF (10 mM) was dissolved in 50 mM sodium phosphate (pD 7.0) prepared with 2H₂O (99.9% isotopic purity). The pD 1; FFCA: 3.1 × 10⁻¹; MMFA: 4.1 × 10⁻¹; and FDCA: 5.0 ± 1. These response factors were used to estimate the mole percentage of each of the compounds in the reactions.

Abbreviations

AAO: aryl-alcohol oxidase; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; DMSO-d₆: deuterium/ammonium/silicone; FDCA: 2,5-furandicarboxylic acid; FFCA: 5-formylfurancarboxylic acid; GC–MS: gas chromatography–mass spectrometry; HPLC: high-performance liquid chromatography; HMWCA: high-molecular-weight furan carboxylic acid; HRP: horseradish peroxidase; kcat: catalytic constant; kcat/Km: catalytic efficiency; Km: Michaelis constant; MMF: 5-methoxymethylfurancarboxylic acid; MMFA: 5-methoxymethylfurancarboxylic acid; MOX: methanol oxidase; PEF: poly(ethylene-furan dicarboxylate); PET: poly(ethylene-terephthalate); TOF: turnover frequency; TON: turnover number; TTN: total turnover number; UPO: unspecified peroxidase.

Authors' contributions

JCarro performed the biocatalytic experiments and wrote the manuscript. EFF produced and purified the UPO used in the experiments. CFA and JCañada performed the NMR experiments. RU and MH discovered UPO. MA engineered the UPO variant for yeast expression. PF and AT designed the experiments and revised the results and manuscript. All the authors revised the final version of the manuscript, and significantly contributed to the discussion and conclusions obtained. All authors read and approved the final manuscript.

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Competing interests

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Ethical approval and consent to participate

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