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Enzyme cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem†

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A one-pot tandem enzyme reaction using galactose oxidase M₃₋₅ and aldehyde oxidase PaoABC was used to convert hydroxymethylfurfural (HMF) to the pure bioplastics precursor FDCA in 74% isolated yield. A range of alcohols was also converted to carboxylic acids in high yield under mild conditions.

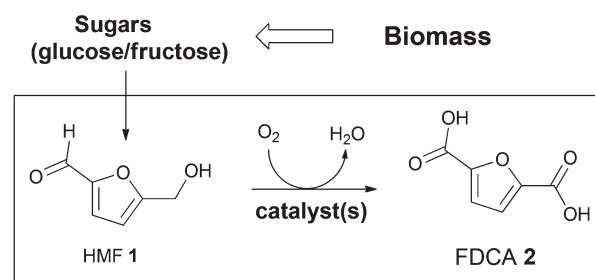
Green chemistry encompasses a set of principles that can be applied in designing sustainable chemical processes. This includes use of renewable raw materials, elimination of waste and avoiding the use of toxic and hazardous reagents and solvents.¹ With the rapid growth of the world population and continuing depletion of petroleum reserves, green approaches using renewable resources for the production of chemicals will be required. Lignocellulosic biomass is an abundant, inexpensive and sustainable resource from which platform chemicals can be derived. 5-Hydroxymethylfurfural (HMF) **1** is derived from cellulose *via* dehydration of glucose and fructose. Due to the instability of HMF, its oxidized and more stable form furan-2,5-dicarboxylic acid (FDCA) **2** is listed as one of twelve sugar-based platform chemicals of interest by the American DOE.²

Polyethylene terephthalate (PET) makes up 5.9% of the global plastics industry with approximately 15 million tons per year being manufactured. It is considered that bio-based **2** could replace terephthalic acid in this and related co-polymers which would be a substantial step towards sustainable plastics manufacture.³ Moreover, **2** is a building block^{2b} and can be used to synthesize other polyesters,⁴ polyamides⁵ and valuable

furanic chemicals.⁶ Obtaining **2** from biomass at low cost will be essential to allow a paradigm shift in green manufacturing although this target has yet to be realized. HMF **1** consists of a furan ring with 2,5-disposed aldehyde and hydroxymethyl functional groups. It can be synthesized from glucose/fructose by dehydration⁷ in high yield although currently only with continuous removal of water or extraction into non aqueous solvents or ionic liquids, due to instability in water at high temperature.^{7d} In order to obtain **2** from **1** a 6 electron oxidation is required. Numerous metal catalysts and nanoparticles have been employed such as Au-TiO₂,⁸ Au-C modified with Pd,⁹ Au-hydrotalcite,¹⁰ Pt-C,¹¹ Au/TiO₂,¹² Pt/ZrO₂,¹³ however these reactions require high pressure/temperature and additives which decreases the sustainability for manufacturing considerably. Thus there remain a number of challenges for the steps from cellulose to FDCA and their integration.

A catalytic system that uses O₂ from air and produces water as the only by-product would contribute to establishing a green and sustainable process for conversion of HMF **1** to FDCA **2** (Scheme 1).¹⁴ To this end, aerobic Pt nanoparticles¹⁵ and gold systems¹⁶ have been developed but usually require high pH which can partially decompose the unstable HMF.

Biocatalytic reactions offer many benefits in the context of green chemistry since they can be performed under mild con-



Scheme 1 Desired route to FDCA from biomass derived sugars.

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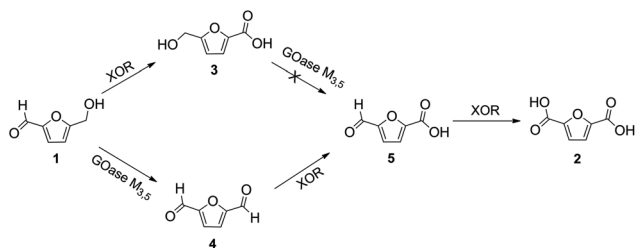
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ditions using a biodegradable catalyst.¹⁷ The synthesis of **2** from HMF has been previously demonstrated using a recombinant whole cell biotransformation.¹⁸ However the need for a continuous carbon source feed and low product recovery of the polar FDCA from the cell biomass limited the potential of this process. Bioconversions using isolated enzymes can proceed at significantly higher substrate concentration and combine higher productivity with a lower water usage. There are only a few examples of bioconversions of **1** to **2** in the literature. A galactose oxidase mutant and peroxidase were used in a tandem reaction to give 46% yield of impure **2** and *Caldariomyces fumago* chloroperoxidase has been reported to incompletely oxidize **1**; both processes use low substrate concentrations (1 mM), require addition of peroxide and result in separation issues.¹⁹ Recently, an FAD-dependent HMF oxidase has been reported to fully oxidize **1** to **2** using molecular oxygen, although at low substrate concentration (2–4 mM).²⁰ Aryl alcohol oxidase (AAO) was used to convert **1** (3 mM) to 5-formylfuran-2-carboxylic acid (FFCA) **5**, then in a second much slower step an unspecific peroxygenase (UPO) converted **5** to **2**.²¹

Recently we have shown that *in vitro* cascades employing two isolated oxidases, GOase M_{3–5} and xanthine dehydrogenase (XDH) quantitatively yield aromatic carboxylic acids starting from benzylic alcohols *via* the intermediate aldehyde.²² We therefore postulated that this methodology could be adopted for the synthesis of **2** from **1**. In the present work we demonstrate a preparative scale (74% isolated yield, 50–100-fold greater [S] than previously reported) for an enzymatic synthesis of **2** from **1** while also expanding the scope for alcohol oxidations under mild conditions. For an enzymatic cascade we envisaged using galactose oxidase M_{3–5}, plus a suitable xanthine oxidoreductase (XOR) for sequential oxidations, ideally in one pot (Scheme 2). One of us previously developed variants of *F. graminearum* galactose oxidase (GOase), such as GOase M_{3–5},²³ that shows a remarkable ability to oxidize secondary and primary benzylic alcohols with H₂O₂ as the only by-product.

Initial studies revealed that **1** was indeed a good substrate for GOase M_{3–5} yielding the dialdehyde **4**. The XOR enzyme should be able to oxidize the aldehyde groups in **4** and **5**, both of which are intermediates (Scheme 2). Four molybdenum-dependent XOR enzymes were chosen to screen for activity



Scheme 2 Possible intermediates *en route* from HMF **1** to FDCA **2** using two enzymes: galactose oxidase M_{3–5} and XOR.

against the available substrates **1**, **4** and **5** (Table S1†). The commercially available *E. coli* XDH, with which we had previously shown oxidation of benzylic aldehydes, was only active with **1**. The *Rhodococcus capsulatus* xanthine dehydrogenase (XDH) single variant E232V²⁴ and double mutant XDH E232 V/ R310 showed activity against all three substrates; however these variants possess very low reactivity with oxygen from air and require an exogenous electron acceptor such as DCPIP for high conversions. One of us²⁵ recently reported an *E. coli* periplasmic aldehyde oxidase (PaoABC) which uses oxygen as the terminal electron acceptor. PaoABC is a 135 kDa heterotrimeric enzyme with a large (78.1 kDa) molybdenum cofactor (Moco)-containing PaoC subunit, a medium (33.9 kDa) FAD-containing PaoB subunit, and a small (21.0 kDa) [2Fe–2S]-containing PaoA subunit.^{25,26} A variety of enzymes have evolved to metabolize aldehydes to less reactive intermediates²⁷ and it is believed that PaoABC plays a role in the detoxification of aromatic aldehydes.²⁵ PaoABC was able to oxidize all three substrates **1**, **4** and **5** and hence this enzyme was selected as the candidate biocatalyst for the aldehyde oxidation step.

A test cascade reaction was set up using **1** with both GOase M_{3–5} and PaoABC present to identify if **2** could be produced. At 10 mM HMF concentration, we were pleased to observe almost complete conversion (97%) to **2** after 1 h, with key intermediates being **4** and **5** (Table S2, entry 1, Fig. S7†). We then investigated the effect of increasing the concentration of **1**. At 20 mM

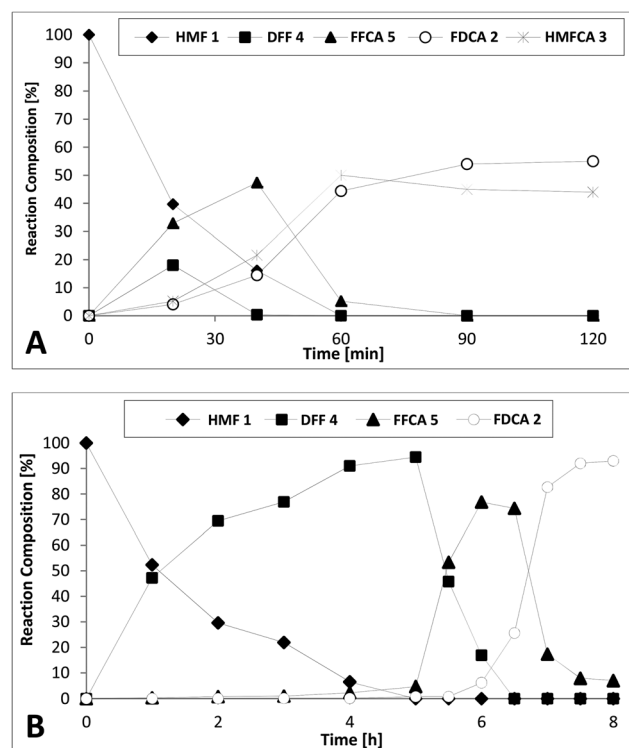


Fig. 1 Enzyme cascades for conversion of HMF (**1**) with (A) dual combined enzymes (GOase M_{3–5} + PaoABC) ([HMF] = 20 mM) and (B) one-pot sequential reaction ([HMF] = 50 mM).

1, after 1 h 2 (44%) was formed along with 3 (50%) (Fig. 1A, Table S2,† entry 2), then after a further 4 h, a small amount of 3 was slowly converted to give a final conversion of 55% of 2. The time course shows that formation of 2 occurs mainly *via* 4 and 5 and that 3 is a poor substrate for GOase M₃₋₅. It is also clear that PaoABC is responsible for the formation of 3 at this substrate concentration.

Production of 3 could be avoided by using a sequential, stepwise process in which the GOase M₃₋₅ conversion of 1 to 4 was allowed to run to completion prior to addition of the PaoABC enzyme (Fig. 1B, Table S2,† entries 3–10). This stepwise reaction furnished the required 2 as the only oxidation product, cleanly and with 100% conversion. Oxidases such as GOase and PaoABC produce H₂O₂ as a byproduct and it was found necessary to remove the peroxide by addition of catalase. Catalase converts H₂O₂ into O₂ and thus provides extra equivalents of O₂ for the reaction, while also protecting the enzymes from oxidative damage.

On increasing the starting concentration of HMF (1) further we found that sufficient buffer capacity to control pH was important to enable the reaction to reach completion (Table S2†). During the optimisation of the conversion of 4 to 2 with PaoABC it was noted that with [S] = 100 mM, not surprisingly a drop in pH was occurring (pH < 5, Table S3,† entry 7), which was below the optimum pH (6–8) of PaoABC, as measured with *m*-anisaldehyde as substrate (Fig. S2†). We believe that this drop in pH is responsible for the reduced conversion, rather than hydrate formation from 5 since although the dialdehyde 4 does form a hydrate at pH 5–8, the aldehyde-acid 5 does not (Fig. S3 and S4†). At 100 mM 1, an additional portion of catalase was required with the addition of PaoABC (Table S2† entries 8 and 9), possibly due to the extensive reaction time for the first oxidation (>16 h), during which time catalase may be deactivated. With our optimized conditions in hand, the preparative scale oxidation of 1 was then realized (Table S2,† entry 10). Isolation of 2 by crystallization has been successful after whole cell biotransformations,¹⁸ however the removal of biomass and numerous extractions into organic solvents reduce sustainability. Using our *in vitro* cascade system, no organic solvents were required. Heat treatment of the solution to precipitate the protein, centrifugation, acidification and filtration is all that was required to obtain pure 2 in 74% isolated yield (ESI 8.0). In the 50 mM sequential reaction (Fig. 1B) the initial oxidation of 1 was found to be the slower step. On addition of PaoABC there is a rapid oxidation of 4 to 5. It is noteworthy that 2 is not produced rapidly until all of 4 is oxidized to 5. The dialdehyde 4 is a better substrate for PaoABC, possibly because its hydrate, which forms rapidly in buffer (Fig. S3†), is the true substrate. In the sequential reaction, the whole oxidation process is complete after 8 h. The hydrate, rather than the aldehyde itself, has recently been reported to be the catalytically active substrate for other oxidase enzymes, including HMF oxidase,²⁰ AAO and other enzymes.^{21,28} In each case the final aldehyde-acid (5) is a poor substrate because the equilibrium for hydrate formation is highly unfavourable. In

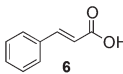
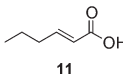
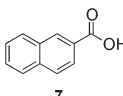
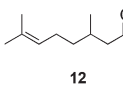
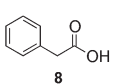
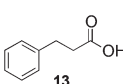
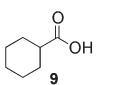
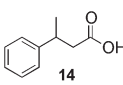
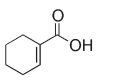
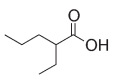
our two-step sequence we cannot completely exclude the possibility that a small fraction of 5 may be produced either directly by GOase M₃₋₅ or by spontaneous oxidation by residual H₂O₂, as a low level of 5 is detected before the addition of PaoABC (Fig. 1B).

Turning our attention back to the combined dual enzyme reaction, we considered that a pH drop might limit oxidation of the hydroxymethyl acid 3 by GOase M₃₋₅. However, oxidation at [S] = 50 mM 1 with GOase M₃₋₅ and PaoABC combined at high buffer concentration resulted in predominantly 3 after 3 h, which was slowly converted to 5. The latter was then rapidly oxidized to 2 and after 17 h, 56% of 2 was present (Fig. S9†).

Having established a tandem process for conversion of 1 to 2, we further explored the substrate specificity of PaoABC for oxidation of a wider range of activated and unactivated aldehydes.

Ten selected aldehyde substrates were shown to be active with PaoABC. Subsequent combination with GOase M₃₋₅ resulted in the successful conversion of the corresponding primary alcohols to carboxylic acids 6–15 (Table 1) with, in most cases, quantitative conversions at 10 mM substrate concentration. A time course study for the conversion of phenylethanol (10 mM) to phenylacetic acid 8 revealed no aldehyde intermediate, indicating that the second PaoABC step was extremely rapid (Fig. S10†). However, increasing the substrate

Table 1 Scope of the GOase M₃₋₅-PaoABC enzymatic cascade^a

$\text{R-OH} \xrightarrow[\text{catalase, O}_2 \text{ (air)}]{\text{GOase M}_{3-5} \text{ buffer pH 7.6, 37}^\circ\text{C}} \left[\text{R-CHO} \right] \xrightarrow[\text{catalase, O}_2 \text{ (air)}]{\text{PaoABC buffer pH 7.6, 37}^\circ\text{C}} \text{R-COOH}$			
Product	Conversion	Product	Conversion
	>99%		>99%
	>99%		>99%
	>99%		>99%
	>99%		>99%
	81%		50%

^a Reaction conditions: 103 μL GOase M₃₋₅ (3 mg mL⁻¹), 5 μL PaoABC (13.2 mg mL⁻¹), 33 μL catalase (1.3 mg mL⁻¹), 3 μL of substrate (1 M in MeCN 10 mM final concentration) in 50 mM pH 7.6 potassium phosphate buffer (159 μL), 37 °C shaking overnight.

concentration to $[S] = 30$ mM caused problems for the GOase M_{3-5} step. For example, using 3-phenylbutan-1-ol required large quantities of GOase to obtain high conversions to **14** (Table S6†). PaoABC showed a remarkable tolerance of high substrate concentration as no aldehyde intermediate could be identified by NMR. Since GOase M_{3-5} currently represents the limiting activity it will be necessary to engineer GOase variants that are more tolerant to higher alcohol concentrations. The GOase M_{3-5} -PaoABC one-pot conversion provides a highly green and direct method for the conversion of alcohols to carboxylic acids and compares favourably with the current approach using a chemocatalytic Ru-pincer complex where reactions are run with 1 equivalent of NaOH in refluxing water.²⁹

Conclusions

In summary we have developed a promising tandem cascade reaction using two oxygen-dependent enzymes, galactose oxidase M_{3-5} and aldehyde oxidase PaoABC, that results in high conversion of **1** to **2** at ambient temperature and near neutral pH. The substrate concentration of **1** (100 mM) is the highest reported for an enzyme-based process and uses enzymes that do not require the addition of diffusible cofactors. Key to the success of this tandem cascade compared with HMF oxidase²⁰ is the ability of PaoABC to efficiently catalyse the final oxidation step of **5** to **2**. Whereas HMF oxidase requires the hydrate of **5**, PaoABC appears to operate on the aldehyde. Further development of the enzymes and reaction conditions, particularly with the aim of interfacing with upstream reactions to produce HMF from cellulose could provide an extremely green and economic route to highly pure FDCA (**2**). We have also demonstrated a one-pot, single stage tandem cascade for the quantitative conversion of 10 alcohols directly to carboxylic acids, including unactivated examples. The conditions are green and compare favorably with state-of-the-art chemocatalytic methods.

Representative procedure for one-pot tandem enzyme reaction for preparation of FDCA (**2**)

HMF (**1**) (38 mg, 0.3 mmol, final concentration = 100 mM) and catalase (0.33 mL of a 3.3 mg mL⁻¹ solution) were added to potassium phosphate buffer (400 mM pH 7) (1.09 mL) and MeCN (0.03 mL). GOase M_{3-5} (1.5 mL of a 3.3 mg mL⁻¹ solution) was then added and the reaction shaken at 37 °C for 10 h in a shaking incubator. After this time, another portion of catalase (0.33 mL of a 3.3 mg mL⁻¹ solution) was added along with PaoABC (0.05 mL of a 13.2 mg mL⁻¹ solution) and the reaction shaken for a further 5 h in the incubator. The pH was carefully monitored and adjusted to pH 7 with 1 M NaOH. After this time the reaction was heated to 80 °C for 5 minutes and left to cool. The solution containing denatured protein

was centrifuged and the supernatant removed. The supernatant was then cooled to 0 °C and concentrated HCl was added dropwise until a precipitate formed. The solution was then centrifuged and the supernatant removed and the pellet washed with 1 M HCl. The pellet was dissolved in acetone and then concentrated *in vacuo* three times yielding **2** as a pale yellow solid (35 mg, 0.22 mmol, 74% yield).

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