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Toward scalable biocatalytic conversion of 5-hydroxymethylfurfural by galactose oxidase using coordinated reaction and enzyme engineering

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5-Hydroxymethylfurfural (HMF) has emerged as a crucial bio-based chemical building block in the drive towards developing materials from renewable resources, due to its direct preparation from sugars and its readily diversifiable scaffold. A key obstacle in transitioning to bio-based plastic production lies in meeting the necessary industrial production efficiency, particularly in the cost-effective conversion of HMF to valuable intermediates. Toward addressing the challenge of developing scalable technology for oxidizing crude HMF to more valuable chemicals, here we report coordinated reaction and enzyme engineering to provide a galactose oxidase (GOase) variant with remarkably high activity toward HMF, improved O₂ binding and excellent productivity (>1,000,000 TTN). The biocatalyst and reaction conditions presented here for GOase catalysed selective oxidation of HMF to 2,5-diformylfuran offers a productive blueprint for further development, giving hope for the creation of a biocatalytic route to scalable production of furan-based chemical building blocks from sustainable feedstocks.

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Biomass waste is an abundant, carbon-rich renewable feedstock that, if processed efficiently, could provide access to many chemicals and fuels as an alternative to those produced from fossil resources. The sugars component of biomass can be chemically dehydrated to give furfural and 5-hydroxymethylfurfural (HMF), of which the latter has been identified by the U.S. Department of Energy as one of the top 12 potential platform chemicals from renewable feedstocks. HMF can be transformed through different reactions to produce a range of derivatives, many having applications in the polymer industry. In particular, the oxidized HMF derivatives 2,5-diformylfuran (DFF) and furan-2,5-dicarboxylic acid (FDCA) are both important intermediates in furan polymer synthesis (Fig. 1), as they can be condensed with other monomers to make poly-imines, -esters, -amides and -urethanes as plastics, resins and porous organic frameworks. In addition to more environmentally responsible manufacturing methods, many of these bio-based plastics have properties that rival those produced from petroleum resources, such as the polyethylene furanoate ‘drop in’ replacement for polyethylene terephthalate, and some even display unique properties of potential use in performance materials.

The primary obstacles preventing the necessary industrial scale production of these furan-based plastics are (1) an inexpensive, continuous and large supply of the basic materials from renewable resources, (2) an efficient and cost effective process to convert them to monomer units, and (3) effective implementation at a production scale that meets the consumer demand. Recent advances in reaction engineering using both inorganic and acid catalysts have enabled high yielding conversions of carbohydrates to HMF. Yet HMF is unstable, and often needs to be purified to remove salts and by-products left as impurities prior to further chemical conversion, in order to avoid catalyst poisoning. Once in hand, HMF can be oxidized by a variety of metal catalysts or electrochemical methods to produce DFF, FDCA, or the dimethyl ester of FDCA (furan dicarboxylic methyl ester, FDME) which are more stable monomers. However, these reactions are frequently performed at elevated temperatures and pressures, which can be energy intensive and therefore counterproductive to the aim of developing a sustainable process.

Preferably, biocatalytic oxidation of HMF to these valuable derivatives could extend the sustainability of these bio-based chemicals to include a more environmentally friendly production process. However, this activity is limited to only a few enzymes, primarily chloroperoxidases, HMF oxidases (HMFO), aryl-alcohol oxidases, and galactose oxidases (GOase) and related copper radical oxidases. The latter three seem to be the most appealing due to their dependence on oxygen as a co-substrate rather than \( \text{H}_2\text{O}_2 \) as in peroxidases or NAD(P) as in dehydrogenases. Nevertheless, this presents a number of issues when working at scale due to limitations in oxygen solubility in aqueous media and thereby supply, that can significantly impact biocatalyst performance. In analogous two-enzyme systems, GOase has been combined with periplasmic aldehyde oxidase PaoABC or unspecific peroxygenases to create an oxidative cascade from HMF to FDCA. However, productivity and selectivity of these systems are not yet adequate for large-scale implementation, and none provide access to DFF as the final product and our desired target. In fact, DFF has been particularly challenging to selectively synthesize efficiently (chemically or biocatalytically) without requiring high levels of (bio) catalysts, but GOase and related copper radical oxidases do provide DFF as the main product.

Building on our previous work in understanding the effect of reaction conditions on GOase for alcohol oxidation (critically, the inclusion of horseradish peroxidase (HRP) to activate GOase and catalase to remove destructive \( \text{H}_2\text{O}_2 \)), we have engineered GOase for improved kinetic properties for both alcohol oxidation and oxygen binding to deliver a variant that exhibits particularly high catalytic activity for HMF oxidation to selectively produce DFF. Coordinated reaction engineering led to further improvements in conversion and demonstrated that a GOase-based synthesis is amenable to the necessary process intensification required for ultimate industrial implementation. This approach comprising coordinated reaction and enzyme engineering has resulted in an effective and high yielding model system for the selective bio-oxidation of HMF to DFF at high substrate loading.

Results and discussion

For DFF, FDCA and their derivatives to be relevant for use in bio-based plastics, the scale of production needs to meet a market demand, and the production process needs to be cost competitive compared to current methods of producing the non-bio-based monomer equivalents. We began by defining a set of proof of principle reaction metric targets to guide our approach based on estimated threshold values for a biocatalytic processes in the bulk chemical sector (Table 1). These metrics were then used to help define which aspects were still in need of improvement before potential future implementation in a true industrial process.

Our approach began with enzyme engineering that was guided by reaction constraints to directly develop a biocatalyst that was industrially relevant, applying screens to select for improvement in turnover rate and for improvements in activity at low oxygen levels. Biocatalyst development was balanced by concurrent, iterative reaction development to tune conditions and push performance limits to identify scalable conditions. This also served as a secondary selection process to identify the top performing variants under potential process conditions. As such, the laboratory scale experiments were performed with the end goal of scale-up in mind. These two approaches proved cooperative, using the results of one to help guide modifications to the other in a so-called ‘ideal scenario’ of process and biocatalyst driven.
process design\textsuperscript{44,47}. In the interests of clarity, work on these approaches is discussed in two separate subsections below, despite being coordinated and occurring simultaneously.

**Enzyme engineering.** The first round of library generation targeted pairs of active site residues (Fig. 2), and screening against 1-hexanol identified variant M\(_4\) (M\(_{2,5} + Y329L/M330F\) mutations) as being the top performing hit (Supplementary Note). Residues outside the active site were also targeted to randomly recombine previously published beneficial mutations into M\(_1\). Mutated sites in variants published by Delagrave et al. (variant 7.5.1 with C383S/Y436H/N318D/V477D/A626S/V494A)\textsuperscript{48}, Wilkinson et al. (variant C383S/Y436H)\textsuperscript{49}, and Deacon and McPherson (C383S and C383T)\textsuperscript{50} were chosen as sites for potential non-selective catalytic improvements. None of these residues interact directly with the substrate (Supplementary Fig. 1), but each variant showed increases in \(k_{cat,app}\), reductions in \(K_{M,app}\), or both. Mutations other than V494A, which was already present in GOase since the M\(_1\) variant (Supplementary Table 1), were randomly introduced to create a library of unique combinations and screened for activity on 1-hexanol. The top hits identified from this screen were variants M\(_{3,1}\) (V477D/A626S), and M\(_{4,2}\) (N318D/C383T/Y436H/V477D). Interestingly, these hits had distinct sequences compared to the previously published variants. Kinetic characterization of these two variants showed very similar parameters compared to the progenitor (Table 2), therefore, it is possible that the mutations have more influence on GOase expression and/or stability, which contributed to their detection in the screen.

A universal issue in the application of oxygen-dependent enzymes revolves around the balance of providing sufficient oxygen to the bioreactor while minimizing potential enzyme deactivation at the gas-liquid interface and potentially stripping volatile substrate(s), product(s) and/or co-solvent.\textsuperscript{33,51}. Oxygen has a low solubility in aqueous solution (~270 µM)\textsuperscript{31,33,52}, while \(K_{M}\) for O\(_2\) (\(K_{M,O2}\)) for many enzymes is often estimated to be much higher than the oxygen concentration in equilibrium with air\textsuperscript{33,53}. This difference means that reaction rates will be dependent upon the oxygen concentration in the liquid and thereby on oxygen transfer rates\textsuperscript{32}. This limitation is magnified at high substrate loadings, where substrate concentration and oxygen concentration can differ by a factor of up to \(10^3\)\textsuperscript{30,32}. The problem results in a system that often prevents the enzyme from operating at maximum performance, and therefore other strategies are required.

One approach to overcome the oxygen problem is to improve the \(K_{M,O2}\) of the biocatalyst, or in other words, engineer the enzyme to work more effectively at lower oxygen concentrations\textsuperscript{32,54–57}. Likewise, improvements in \(k_{cat}/K_{M,O2}\) (catalytic efficiency for oxygen) will be more beneficial than improvements in \(k_{cat}/K_{M}\) (where \(K_{M}\) is the \(K_{M}\) for the target substrate). Because the active site of GOase is fairly exposed on the surface of the enzyme, residues nearest to the active site Cu\(^{2+}\) ion were expected to have the greatest impact on oxygen binding and reactivity. The solid phase assay proved to be amenable for use within a glovebox, allowing the screen to be performed in a controlled environment containing a low O\(_2\) atmosphere (around 0.2% v/v, see Supplementary Note). Using this format, active site libraries C, D, and E (Fig. 2) on the GOase M\(_4\) template were screened for improved GOase activity at low oxygen levels using HMF as substrate. Despite the low oxygen concentration, multiple potential hits from libraries C and D were identified. Upon confirmation in a secondary screen and basic characterization in analytical scale biotransformations to challenge these newest variants in current reaction conditions (see below), GOase M\(_{6-A}\) (F290W/S291S) was identified as the top performing variant. Additionally, one potential hit from library C (variant M\(_{6-L}\), with F290W/S291R mutations) was identified after quickly producing a uniquely large spot on the assay plate following removal after several hours of incubation at the low oxygen atmosphere. The common mutation between these two variants is the reversion of F290 to tryptophan, which is the residue found in the wild-type enzyme and the early GOase M\(_1\) variant\textsuperscript{58}, but was mutated to phenylalanine during engineering of the GOase M\(_1\).
variant\textsuperscript{59} (Supplementary Table 1). It appears that W290 has a significant impact on substrate (HMF and/or O\textsubscript{2}) binding and catalysis since the $k_{\text{cat}}$/M for HMF in the M\textsubscript{6-A} variant increased by 50\%, while the $K_{\text{M}}$ increased by approximately 5-fold (Table 2). The $K_{\text{M}}$ for M\textsubscript{6-B} was similarly affected, although there was little change in $k_{\text{cat}}$/M, indicating that the S291R mutation counteracts the beneficial effect of the F290W mutation in this variant.

The final round of enzyme engineering was based on merging the results of the two evolution strategies to create the M\textsubscript{7-1A}, M\textsubscript{7-2A}, M\textsubscript{7-1B}, and M\textsubscript{7-2B} variants (Supplementary Table 1). M\textsubscript{7-1A} and M\textsubscript{7-2A} were kinetically characterized, revealing an increase in $k_{\text{cat}}$/M to 246 s\textsuperscript{-1} for M\textsubscript{7-1A} (Table 2), which compares quite favourably to FAD-dependent enzymes that oxidize HMF\textsuperscript{24-26,36,37}, and is similar to another copper radical oxidase variant\textsuperscript{37}. In this instance, the combination of mutations from M\textsubscript{5-1} and M\textsubscript{5-2} with M\textsubscript{6-A} appears to be additive, which is a reflection of the non-specific catalytic enhancement provided by such distal mutations.

The kinetic measurements above are apparent values since they were determined under oxygen limited conditions. A more complete dataset was collected for selected GOase variants using the recently developed Tube-in-Tube Reactor (TITR),\textsuperscript{60} which has enabled a simplified method for accurately determining true kinetic parameters for oxygen-dependent enzymes, specifically $K_{\text{M}}$\textsubscript{O}.\textsuperscript{33} The variants chosen for these detailed characterizations were selected to sample the improvements over the course of the engineering program in comparison to the wild-type (M\textsubscript{1}) variant: the initial M\textsubscript{3} progenitor, the first hit for improved HMF activity (M\textsubscript{4}) and the top hit from the low O\textsubscript{2} screen (M\textsubscript{6-A}).

$K_{\text{M}}$\textsubscript{O} values changed significantly over the course of evolution, increasing drastically in early variants before returning to wild-type levels after screening in the low oxygen environment (Table 3). The influence of $K_{\text{M}}$\textsubscript{O} can be observed by comparison of the TITR data with the apparent kinetic parameters reported in Table 2. Measurements for the M\textsubscript{4} and M\textsubscript{6-A} variants are fairly consistent between the two experiments as a result of $K_{\text{M}}$\textsubscript{O} values near or below the level of aqueous O\textsubscript{2} concentration in equilibrium with air (~270 µM and 30 °C). However, the high $K_{\text{M}}$\textsubscript{O} for GOase M\textsubscript{5-1} and M\textsubscript{5-2} with M\textsubscript{6-A} appears to be additive, which is a reflection of the non-specific catalytic enhancement provided by such distal mutations.

### Table 3 Kinetic parameters of GOase variants determined in the TITR with HMF.

| Variant | $K_{\text{M}}$\textsubscript{O} (mM) | $K_{\text{M}}$\textsubscript{S} (mM) | $k_{\text{cat}}$ (s\textsuperscript{-1}) | $k_{\text{cat}}$/M\textsubscript{O} (M\textsuperscript{-1}s\textsuperscript{-1}) | $k_{\text{cat}}$/M\textsubscript{S} (M\textsuperscript{-1}s\textsuperscript{-1}) |
|---------|------------------|------------------|-----------------|-----------------|-----------------|
| M\textsubscript{1} | 0.16 ± 0.11 | 53 ± 18 | 23.5 ± 4.7 | (1.5 ± 1.1) × 10\textsuperscript{5} | (0.04 ± 0.02) × 10\textsuperscript{4} |
| M\textsubscript{3} | 1.39 ± 0.46 | 14.9 ± 4.5 | 651 ± 132 | (4.7 ± 1.8) × 10\textsuperscript{5} | (4.4 ± 1.6) × 10\textsuperscript{4} |
| M\textsubscript{4} | 0.37 ± 0.07 | 1.83 ± 0.30 | 120 ± 6 | (3.3 ± 0.6) × 10\textsuperscript{5} | (6.6 ± 1) × 10\textsuperscript{4} |
| M\textsubscript{6-A} | 0.15 ± 0.02 | 6.19 ± 0.41 | 166 ± 4 | (10.9 ± 1.5) × 10\textsuperscript{5} | (2.7 ± 0.2) × 10\textsuperscript{4} |

$k_{\text{cat}}$/M\textsubscript{O} for oxygen, $k_{\text{cat}}$/M\textsubscript{S} for HMF. \textsuperscript{26,36,37} Measured using CFE powder with an estimated GOase content, giving a higher uncertainty in the $k_{\text{cat}}$/parameter. Data previously published in the PhD thesis of A. T. P.\textsuperscript{77}.
Table 4 Conversion of HMF to DFF by GOase variant M₄ CFE using EtOAc overlay at reduced incubation temperature.

| Entry | GOase Variant | GOase (g/L) | GOase Form | HRP (g/L) | [HMF] | Type HMF | Conversion (%) | Over-oxidation (%) |
|-------|---------------|-------------|------------|-----------|-------|----------|---------------|--------------------|
| 1     | M₄            | 0.625       | CFE        | 0.0128    | 250 mM| Pure     | 96            | 2.5                |
| 2     | M₄            | 0.625       | CFE        | 0.0128    | 25 g/L| Crude    | 88            | 6.4                |
| 3     | M₄            | 0.625       | CFE        | 0.0128    | 500 mM| Pure     | 71            | 0.4                |
| 4     | M₄            | 0.625       | CFE        | 0.0128    | 50 g/L| Crude    | 82            | 0.8                |

Data for reactions with purified enzyme provided in Supplementary Table 10. Conditions listed in the table highlight the main differences between samples, while those listed below the table apply to all entries unless otherwise stated.

Conditions: 0.05 mM CuSO₄ in GOase M₄ CFE reactions, 880 U/mL Catalase, 100 mM NaPi, 200 µL (80% of aqueous volume) EtOAc, 20 °C, 6 h.

Table 5 Conversion of HMF to DFF by GOase variant M₄ CFE at high HMF loading in the presence of different cosolvents.

| Entry | GOase Variant | GOase (g/L) | GOase Form | HRP (g/L) | Co-Solvent | [HMF] (g/L) | Type HMF | Conversion (%) | Over-oxidation (%) |
|-------|---------------|-------------|------------|-----------|------------|-------------|----------|---------------|--------------------|
| 1     | M₄            | 0.625       | CFE        | 0.0128    | EtOAc      | 50          | Crude    | 82            | 0.8                |
| 2     | M₄            | 0.625       | CFE        | 0.0128    | DEC        | 50          | Crude    | 82            | 0.8                |
| 3     | M₄            | 0.625       | CFE        | 0.0128    | BuOAc      | 50          | Crude    | 76            | 0.7                |

Data for reactions with purified enzyme provided in Supplementary Table 11. Conditions listed in the table highlight the main differences between samples, while those listed below the table apply to all entries unless otherwise stated.

Conditions: 0.05 mM CuSO₄ in GOase M₄ CFE reactions, 880 U/mL Catalase, 100 mM NaPi, 200 µL (80% of aqueous volume) solvent overlay, 20 °C, 6 h.

Integrating these two beneficial reaction features (i.e. 20 °C and EtOAc layer) gave an improved set of performance metrics: almost complete conversion of 250 mM HMF and 25 g/L crude HMF by GOase M₄ as purified enzyme and as CFE after 6 h, suggesting that substrate loading could be pushed still higher (Table 4 and Supplementary Table 10). Indeed, under the same conditions, conversion to DFF reached 71% of 500 mM HMF and 82% of 50 g/L crude HMF by M₄ CFE after 6 h.

Large scale application of GOase will likely require a continuous supply of air (or oxygen enriched air) to meet the oxygen requirements for substrate conversion. Consequently, low boiling solvents, such as EtOAc (bp 77 °C), would be stripped out of the reaction vessel, even with the installation of an effective condenser. We therefore evaluated organic solvents with lower vapour pressures but with log P values similar to EtOAc (Supplementary Note, Supplementary Fig. 2). Co-solvents that proved to be more beneficial and compatible with GOase were esters, carbonates and ethers, with diethyl carbonate (DEC, bp 126 °C) identified as the most promising (for unfavourable solvents, see Supplementary Note). DEC was found to give comparable levels of conversion at both 250 and 500 mM unfavourable solvents, see Supplementary Note). DEC was found

Table 6 Conversion of 100 g/L semi-crude HMF to DFF by GOase variants.

| Entry | GOase Variant | Conversion at 6 h (%) | Conversion at 24 h (%) |
|-------|---------------|-----------------------|-----------------------|
| 1/2   | M₄            | 31                    | 31                    |
| 3/4   | M₅-1          | 38                    | 39                    |
| 5/6   | M₅-2          | 51                    | 54                    |
| 7/8   | M₆-2A         | 57                    | 62                    |
| 9/10  | M₆-2B         | 59                    | 76                    |
| 11/12 | M₇-1A         | 64                    | 69                    |
| 13/14 | M₇-2A         | 77                    | 77                    |
| 15/16 | M₇-2B         | 64                    | 81                    |
| 17/18 | M₇-2B         | 58                    | 78                    |

Conditions: 0.05 g/L pure enzyme, 0.0064 g/L HRP, 880 U/mL Catalase, 100 mM NaPi, 100 g/L Crude HMF in DEC (BASF), 200 µL (80% of aqueous volume) solvent overlay, 20 °C, 6 and 24 h. Over-oxidation in all samples was found to be <0.3%.
variants, GOase M7-2A was consistently the most productive in all gDFF/genzyme (Supplementary Table 2 Entry 7).

biocatalysts63 evaluate the top performing GOase variants (M6-B,M7-2A,M7-1B and M7-2B) challenged with 150 g/L (~1.2 M) semi-crude HMF in solvent (BASF), 200 µL (80% of aqueous volume) solvent overlay, 20 °C, 24 h. No over-oxidation was observed in any of the samples. DEC, diethyl carbonate. DEM, diethyl malonate. DES, diethyl succinate. EGDA, ethylene glycol diacetate. Each bar represents a single experiment.

several other co-solvents that offered slightly different physical properties (density, Log P, water solubility, boiling point, etc., Supplementary Table 14) were identified for compatibility testing with GOase. Of these solvents, diethyl malonate, diethyl succinate, and ethylene glycol diacetate (DEM, DES and EGDA, respectively) gave similar conversions as the standard reaction with DEC at 100 g/L semi-crude HMF, while all others gave significantly lower conversion (Supplementary Fig. 3). These four solvents (DEC, DEM, DES and EGDA) were then used to evaluate the top performing GOase variants (M6-B, M7-2A, M7-1B and M7-2B) challenged with 150 g/L (~1.2 M) semi-crude HMF (Fig. 3). Despite the apparent stability improvements of the ‘B’ variants, GOase M7-2A was consistently the most productive in all reaction compositions, reaching up to 66% conversion after 24 h with DEM as the co-solvent, remarkably approaching 100 g/L product concentration.

A calculation of total turnover number under these conditions with 0.05 g/L (0.71 µM) purified GOase translates to an exceptionally high total turnover number (TTN) of 1.11 × 10⁶ for the GOase M7-2A variant in DEM, giving an excellent comparison to TTN ranges for other industrially applicable biocatalysts63–67. The highest productivity though was achieved with GOase M7-2A at 100 g/L semi-crude HMF in DEC after 6 h (Table 6 Entry 13), reaching 12.8 g/L h (aqueous volume only, or 7.1 g/L when including solvent volume) and a specific yield of 1500 gDFF/genzyme. This represents a nine-fold increase in productivity with a seven-fold increase in specific yield when compared to the M3-5 progenitor enzyme at 1.4 g/L h and 218 gDFF/genzyme (Supplementary Table 2 Entry 7).

In an effort to increase HMF conversion at high substrate loadings (100 and 150 g/L semi-crude HMF in DEC), reactions were performed with 0.1 g/L GOase M7-2A. After 6 h, 100 g/L HMF was almost fully converted to DFF (96%), while conversion of 150 g/L loading lagged behind (62%) (Supplementary Table 15). In both cases extended reaction times led to little further increase in conversion.

**Preliminary reaction testing.** Relatively successful preliminary 10 mL scale reactions during early stage reaction engineering provided a benchmark against which to measure reactions at larger scale (Supplementary Table 16). GOase variants M3-5, M6-A and M7-2A were selected to highlight the improvements in performance gained through the low oxygen screening conditions (M6-A) and recombined best mutations (M7-2A) compared to the initial progenitor (M3-5).

Conditions for the 0.2 L reactions were evaluated to set initial parameters to allow comparison of the variants (Supplementary Note). After 6 h, GOase M3-5 reached 24% conversion of 50 g/L HMF, while GOase M6-A and M7-2A had similarly improved performance at 37 and 36% conversion, respectively (Supplementary Fig. 4a and Supplementary Table 17 Entries 1–3). This conversion was lower than expected from the analytical scale reactions, which we surmised was at least partly due to the very low (~50 U/mL) catalase loading compared to the analytical scale reactions (440 or 880 U/mL) not being able to convert H₂O₂. Indeed, when the catalase concentration was increased in reactions using M7-2A, up to 59% conversion was observed after 6 h (Supplementary Fig. 4b and Supplementary Table 17 Entries 4–5) indicating that catalase activity was a limiting factor. Additional complications were observed in the transition to this scale, with a significant amount of DEC lost due to stripping in the bubbled reactor, and the formation of emulsions, both of which would likely influence biocatalyst performance.

By comparison to the best analytical scale reactions, the 0.2 L reaction at high catalase loading gave a volumetric productivity of 4.8 g/L h (aqueous volume only, or 2.6 g/L h when including solvent volume) and specific yield of 27.3 gDFF/gbiocatalyst as CFE, or 516 gDFF/gbiocatalyst as pure enzyme equivalent (Supplementary Table 17). As an additional separate validation at scale, a version of this biphasic reaction was performed by BASF at a total volume of 1.44 L (0.8 L aqueous, 0.64 L DEC) using 0.05 g/L GOase with 31.5 g/L HMF loading. Here, the reaction resulted in 92% isolated yield of DFF (at a specific yield of 570 gDFF/gbiocatalyst) demonstrating the feasibility of this reaction design.

The results presented here highlight the value of harmonizing informed reaction and process considerations into the enzyme engineering workflow to directly develop a biocatalyst that balances process and enzyme requirements. Using this coordinated approach to reaction and enzyme engineering, we have developed an advanced GOase variant and corresponding reaction conditions for effective production of the bio-based chemical building block DFF that could be used directly in further enzymatic (or chemo-catalytic) transformations28,68,69 to produce other key bio-derived furans for industrial applications. The low catalyst loading of our GOase M7-2A variant combined with the achievement of high conversion and TTN at >1 M substrate concentrations is unique for biocatalysts active on HMF, and is additionally distinctive in its selective oxidation to the dialdehyde product. To the best of our knowledge, this engineered GOase and the associated reaction conditions represent the most efficient and selective biocatalytic production of DFF to date, and certainly compares favourably with available chemo-catalytic synthetic methods32,40,41.

Despite these advances, there are still several aspects that need further work to reach and surpass the performance metrics outlined in Table 1, in addition to a variety of considerations for translation to industrial scale. For instance, further improvements in enzyme stability are necessary to enable high conversion at increased substrate loadings to elevate production titres to meet expected production scale demands. These could potentially be found through additional enzyme engineering to improve aldehyde tolerance and biocatalyst lifetime, for example, but ultimately process development at a scale that would allow in situ product removal (ISPR)70 is likely required to bring productivity and specific yield into a more viable range. Additionally, replacement of HRP as GOase activator with a chemical71 or

![Fig. 3 Solvent screening for improved conversion at high HMF loading.](Image)

GOase variants were evaluated for conversion of 150 g/L semi-crude HMF to DFF in the presence of different cosolvents. Conditions: 0.05 g/L pure enzyme, 0.0064 g/L HRP, 880 U/mL Catalase, 100 mM NaPi, 150 g/L Crude HMF in solvent (BASF), 200 µL (80% of aqueous volume) solvent overlay, 20 °C, 24 h. No over-oxidation was observed in any of the samples. DEC, diethyl carbonate. DEM, diethyl malonate. DES, diethyl succinate. EGDA, ethylene glycol diacetate. Each bar represents a single experiment.
electrochemical oxidation can provide a cheaper and equally inexpensive and reliable method for HMF synthesis. Electroweave oxidation may provide a cheaper and equally inexpensive and reliable method for HMF synthesis.

Pichia established GOase variant presented here, presumably based on the well-known GOase expression systems for GOase. Biocatalyst production in this way may also, at least partially, alleviate foaming issues due to a purer biocatalyst formulation, as was observed in the validation reaction by BASF. Furthermore, methods for bulk biocatalyst production may provide an initial rate between 0.2–0.5 mmol/L/min. Pumps were primed with the appropriate stock solution, and then set to run continuously to reach a steady state. The mass-flow controllers were set to produce a defined mixture of oxygen and nitrogen, and the system was set to wait a total of three residence times for all set-points to ensure steady state had been reached. After this, the sample was injected, and the ramp method was started. The ramp method gradually lowers flow rate from the initial value to increase the residence time and changing the inlet flows in the reactor. The reactor output provides precise time series data for the experiment. The slope of the residence time ramp, was set to 0.5 for all experiments reported here, and the ramp in flow was run for 15 min (real time) to provide data from a residence time of 2 min to 10 min. Samples were collected every minute to provide 11 samples for each experiment, and initial rates for each were determined. The per cent oxygen in the gas and the flow rate for each syringe pump were all controlled by LabVIEW, which automatically adjusted the set-point according to a predefined list of set-points for the experiment.

Sixty seconds after injection, the detector was balanced and zeroed. Once the system stabilized and reported as ready, the injection valve was turned to inject the sample via the flowing mobile phase. Spectral data was collected over time (20 Hz frequency) to identify mutants. Afterward, a 10 mL solution of reaction mix containing 100 mM 1-hexanol, 0.8 mM CuSO4.5 H2O, 0.1 g/L HRP and 4 mM 4-chloronaphthol in 100 mM NaPi, pH 7.0 with 20% DMSO was combined with 10 mL 2% agarose solution and poured over the membrane to begin the assay. Approximately 3000 colonies per library were screened, with colonies that turned dark purple indicating activity on 1-hexanol. Positive clones were picked from the corresponding replicated membrane the next day, and grown overnight in 5 mL LB supplemented with kanamycin. To confirm hits, a small aliquot of the overnight culture was diluted in LB, re-streaked onto nylon membranes (Roche, 82 mm) on LB agar containing 50 µg/mL kanamycin and grown at 26 °C for 40 h. Colonies were permeabilized and assayed as described above combining 5 mL of both reaction mix and agarose solutions to pour over the membrane. DNA from the top hits from the screen (i.e. those changing colour the fastest) was collected via MiniPrep (Qiagen) from the remainder of the 5 mL overnight culture and submitted for sequencing to identify mutations.

This assay was also performed with 25 mM 1-hexanol alcohol to screen approximately 1000 colonies of the multisite mutagenesis library created on GOase M1 for each substrate.

### Methods

#### Materials

All reagents used were of the highest grade available from Sigma-Aldrich or Fisher Scientific and used without further purification. Libraries on the GOase M5, M6 template in pET30 were synthesized by DNA2.0 to have NNN degeneration at codon positions corresponding to the previously published GOase libraries A, B, C, D, E, F and G. Mutagenic primers were purchased from MWG Eurofins. Cell free extract (CFE) powders were made from PROKOD prep kit unless otherwise stated. A codon optimization version of GOase M5–M6 was used for production of M5–M6 CFE for the scale up biotransformation due to poor expression of this variant. Our standard GOase M5–M6 and GOase M5–M6 constructs were used for the other preparations. The sequence was optimized by IDT, synthesized by LifeTechnologies and cloned into pET30 at Nhel/Xhol sites using NEBuilder according to the manufacturer’s protocol.

#### Galactose oxidase expression and purification

Galactose oxidase variants were expressed and purified as previously described. The PET30 construct of GOase variant was transformed into BL21 Star (DE3) cells (Invitrogen) according to manufacturer’s protocol and grown overnight at 37 °C on LB agar plate containing 30 µg/mL kanamycin. A single colony was picked to inoculate an overnight culture of 5 mL LB supplemented with 30 µg/mL kanamycin and grown at 37 °C. 300 µL of this overnight culture was used to inoculate 250 mL of 8Y2–LAC-SUC auto-induction media29 and grown at 26 °C for approximately 64 h. Cells were harvested by centrifugation at 2800 g for 40 min at 4 °C. The cell pellet was resuspended in 25 mL of buffer (50 mM PIPE, 25% sucrose (w/v), 5 mM MnCl2, 1% Triton X-100 (v/v), and 1 mg/mL lysozyme and gently shaken at 4 °C for 20–30 min before being lyzed by sonication (20 s on, 20 s off, 2 cycles). The clarified lysate (centrifuged 39000 × g for 30 min at 4 °C) was transferred to 30 kDa cut-off dialysis tubing and dialyzed into NP buffer (50 mM NaPi, 300 mM NaCl, pH 8.0) overnight with stirring at 4 °C. The dialyzed sample was passed through a 0.45 µm syringe filter and applied to one or two (in series) 5 mL C-Trap Superflow Plus columns (equilibrated with NP buffer) using a peristaltic pump. The column flow through was collected and applied to the column a second time. After loading the crude lysate, the column was washed with 5 CV of NP buffer followed by elution with 70 mL of NPD buffer (NP buffer with 5 mM desthiobiotin) collecting ~5 mL fractions. Fractions containing GOase (as determined by SDS gel) were combined and concentrated using a Vivaspin before being aliquoted and frozen in liquid N2 prior to storage at –80 °C.

#### Mutagenesis methods

Site-directed mutagenesis was performed following the manufacturer’s protocol from the QuikChange II Site-Directed Mutagenesis kit using Phusion (NEB) polymerase. Multisite-directed mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent) according to the manufacturer’s protocol. Primers used in the protocols are listed in Supplementary Table 19.

#### Solid phase screen on benchtop

The solid phase GOase library screen was performed similarly to that previously published. The library plasmid pool was transformed into chemically competent BL21Star(DE3) E. coli and spread onto a nylon membrane (Roche, 132 mm) on LB/agar containing 50 µg/mL kanamycin. After growth at 26 °C for 40 h, the library was replicated onto a new membrane and grown at 37 °C overnight on a fresh LB/agar/kanamycin plate while the original membrane was used for the assay. Colonies on the membrane were permeabilized by incubation in a chloroform vapour chamber for 10 min at room temperature.
collect 3-dimensional data of time-wavelength-absorbance. Absorbance was measured from 210 to 600 nm with a slit width of 4 nm and a step width of 1 nm. The solutions were made as listed above for characteristic separation of the GOase variants. The solution containing the GOase variant was pumped at a constant ratio of 1/5 of the total flow rate through the reactor, while the flow rate of buffer and HMF solutions were varied according to the HMF concentration set-point. Gas flow was kept constant at 1 NL/min, while the volume fraction of oxygen was varied from 5%–100%. The reactor was pressurized to 1 bar to provide sufficiently high oxygen concentrations in the reaction solution.

Data handling and parameter estimation was performed using MATLAB (MathWorks, Natick, MA, USA). Raw data from LabVIEW were converted to concentration of HMF and DFF using a calibration model. A linear model was fitted to the concentration-time data to determine initial rate as slopes, and the data was discarded if the model did not describe the data sufficiently well ($R^2 < 0.9$). Parameters of the enzyme kinetic model (ping pong bi bi) were estimated using non-linear least square regression and including the uncertainty of the initial rate data. Because the UV absorbance spectra of HMF and DFF overlap, multivariate data analysis was needed to determine the substrate and product concentrations. To do this, a chemometric model was made and calibrated using the PLS Toolbox (Eigenvector Research, Manson, WA, USA) for MATLAB. Calibration curves using for HMF and DFF using this model are shown in Supplementary Fig. 5.

Analytical Scale biotransformation and HPLC analysis. The general analytical scale biotransformation (250 µL) contained 0.05 µL purified GOase variant, 1.7 µL of 50 µM NaPi buffer, then CuSO4 and HRP were added, mixed and allowed to stand for 15 min method with the same conditions. Retention times are 23.0 min for HMF, 26.9 min for DFF, 6.1 and 4.4 min, respectively and 10.5 and 8.2 min, respectively, in Method 2. Concentrations were determined by comparison of peak area to a standard curve. Supplementary Fig. 7 shows the calibration curves for HMF and DFF.

From the aqueous fraction, a 10 µL aliquot was combined with 75 µL acetonitrile and 415 µL H2O and then centrifuged and transferred to a filter vial for analysis by HPLC as described above.

10 mL scale biotransformations. The general scaled up biotransformation (10 mL) contained 0.05 µL purified GOase variant, 1.7 µL of (0.0064 µL) HRP, 880 µL of (0.08 µL) catalase, and 250 mM HMF in 100 mM NaPi pH 7.4. Deviations from this are described in the text and include changes in enzyme loading, enzyme formulation, miscible or immiscible cosolvent, substrate loading, substrate purity, buffer strength or the addition of CuSO4. After addition of HMF to start the reaction, tubes were shaken at 250 rpm at the temperatures described for 0.5 mL scale biotransformations combining aliquots typically up to 1.5 mL, occasionally up to 8 h) to refresh oxygen in the headspace if no time point sample was needed at the given duration.

Aliquots (10 µL) from reactions with water miscible solvents were removed at hourly time points (1–8 h) and after 24 h and quenched by addition of 150 µL acetonitrile and 10 µL 0.5 M H2SO4 before addition of 830 µL H2O. Samples were centrifuged and transferred to a filter vial for analysis. Samples were analysed by HPLC as described above. Reactions with water immiscible solvents were quenched by addition of 1.5 mL 0.5 M NaH2SO4. The sample was vortexed, centrifuged, and the organic layer was removed to dry over Na2SO4. The aqueous phase was extracted twice with 20 mL ethyl acetate and combined. Organic layers over Na2SO4. From the aqueous fraction, a 10 µL aliquot was combined with 37.5 µL acetonitrile and 202.5 µL H2O and then centrifuged and transferred to a filter vial for analysis. A 250 µL aliquot of the organic layer was dried by GeneVac, and the resulting solid was dissolved in 750 µL acetonitrile. The sample was prepared for analysis by combining 37.5 µL of this with 75 µL acetonitrile and 10 µL 0.5 M H2SO4 then diluted to 1500 µL before analysis.

Reactions with water immiscible solvents were quenched by addition of 100 µL of the corresponding solvent (200 µL) contained 0.05 g/L puri ed GOase variant, 1.7 U/mL (0.0064 µL) HRP, 880 U/mL (0.08 µL) catalase, and 250 mM HMF in 100 mM NaPi pH 7.4. Deviations from this are described in the text and include changes in enzyme loading, enzyme formulation, miscible or immiscible cosolvent, substrate loading, substrate purity. After addition of HMF to start the reaction, tubes were shaken at 250 rpm at the temperatures described, however, 20 °C was typically used. Reactions were opened every hour (up to 8 h) to refresh oxygen in the headspace if no time point sample was needed at the given duration.

Aliquots (10 µL) from reactions with water miscible solvents were removed at hourly time points (1–8 h) and after 24 h and quenched by addition of 150 µL acetonitrile and 10 µL 0.5 M H2SO4 before addition of 830 µL H2O. Samples were centrifuged and transferred to a filter vial for analysis. Samples were analysed by HPLC as described above.

Production of CFE for 0.2 L reactions. GOase variants M3, M4, and codon optimized M24 (M24 s) were transformed and expressed as described above. Harvested cell pellets were resuspended in 50 mM NaPi pH 7.0 (5 mL per 1 g cell paste) and sonicated at 1 min on (16 amps), 1.5 min off for 6–8 cycles. The suspension was centrifuged at 39,000 × g for 30 min at 4 °C (Beckman Coulter Avanti J-E), and the clarified lysates were flash frozen in liquid N2 and lyophilized. SDS gel analysis of the CFE preparations indicates similar levels of expression for all three variants (Supplementary Fig. 8).

Specific activities of GOase CFE. Specific activity for the three CFE preps was performed as described above. Kinetics, with 0.05 mM CuSO4 in the reaction mix at 25 mM HMF (final concentration) as substrate. Initial rates were normalized to concentration of CFE in the reactions and are presented in Supplementary Table 18.

0.2 L scale reactions. All 0.2 L scale biocatalytic reactions were performed in a 250 mL bioreactor (MiniBio with my-Control from Applikon Biotechnologie, Delft, Netherlands). The reactor was equipped with a metal sparger, two Rushton turbines, temperature control, a dissolved oxygen (DO) sensor to monitor stripping of the organic solvent layer. Samples were analysed on a Thermo Fisher Scientific Dionex Ultimate 3000 HPLC with a Diode Array Detector. Analytes were separated on a Bio-Rad Aminex HPX-87H column at 60 °C using an isocratic method of 15% acetonitrile/85% 5 mM sulfuric acid as mobile phase. HMF and DFF were monitored at 260 nm.

All the concentrations refer to the concentration in the aqueous phase. Reactions were performed using 100 mM NaPi pH 7.4 as buffer and DEC as cosolvent, 0.05 mM CuSO4, and 4 U/mL HRP, with the temperature kept consistent at 20 °C for all experiments. Concentrations of GOase CFE, HMF and catalase, in addition to stirring speed and aeration rate were varied as indicated to optimize performance and compare activity of GOase variants.

Reactions were performed at a working volume of 200 mL total, split between 110 mL aqueous phase and 90 mL organic phase. Catalase was added to 109 mL with 37 µL of the reactor solution (0.5 µL/10 mL of HMF in DEC). A temperature sensor was added and a dissolved oxygen (DO) sensor was calibrated then added to the reactor. The DO sensor was used to monitor the activity of GOase over the reaction. The reactor was set to the desired experimental parameters (temperature, stirring speed, air flow rate into the reactor) and allowed to equilibrate. In a separate tube, the specified amount of GOase variant CFE was resuspended in 1 mL NaPi buffer, then CuSO4 and HRP were added, mixed and allowed to stand for 1–2 min to activate GOase before transferring the solution to the reactor to start
the reaction. The amount of G0ase CFE used in each experiment was adjusted to be equivalent to 0.05 g/L purified enzyme based on comparison of specific activity of the corresponding CFE protein.

Samples of the reaction mixture (1 mL) were taken at the indicated timepoints via syringe. The sample was vortexed then 950 µL was transferred to a new tube containing 50 µL 2 M sulfuric acid and vortexed to quench the reaction. After filtering (0.45 µm filter) the sample to remove emulsions and separating the phases, 10 µL was taken from both the organic and aqueous phases and each diluted with 427.5 µL 15% acetonitrile in water and 62.5 µL 2 M sulfuric acid. A portion of this sample was transferred to an HPLC vial for analysis on HPLC (5 µL injection) as described above.

**Reporting summary.** Further information on research data is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source data are provided with this paper. Received: 9 December 2020; Accepted: 6 July 2021; Published online: 16 August 2021

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Author contributions

All authors contributed to the planning of the work. W.R.B. performed enzyme engineering, library screening, enzyme production and purification, kinetic characterization, and small scale biotransformations. A.T.P. performed enzyme characterization by TiTR and solvent vapour pressure calculations. M.D.G. and M.B.M. performed 0.2 L scale reactions. M.B. provided substrate formulations for use in reactions. I.M.W. and N.J.T. supervised the work. Initial draft of the manuscript was written by W.R.B. with subsequent contributions from A.T.P., M.D.G., M.B.M., I.M.W. and N.J.T.

Competing interests

The authors declare no competing interests.

Additional information

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