Biocatalytic conversion of 5-hydroxymethylfurfural by galactose oxidase: Toward scalable technology using integrated process and enzyme engineering

William R. Birmingham[a], Asbjørn Toftgaard Pedersen[b,d], Mafalda Dias Gomes[b], Mathias Bøje Madsen[b], Michael Breuer[c], John M. Woodley[b], Nicholas J. Turner[a]*

[a]School of Chemistry, The University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, Manchester, M1 7DN, UK

[b]Department of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[c]BASF SE, White Biotechnology Research, RBW/OB-A030, 67056 Ludwigshafen, Germany

[d]Current Address: Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark

Abstract

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. To address the challenge of developing scalable technology for oxidizing crude HMF to more valuable chemicals, we have integrated process and enzyme engineering to provide a galactose oxidase (GOase) variant with remarkably high activity toward HMF, improved O_2 binding and excellent productivity (>1,000,000 TTN). The process concept presented here for GOase catalysed selective oxidation of HMF to 2,5-diformylfuran offers a productive and efficient platform for further development, thereby laying the groundwork for a biocatalytic route to scalable production of furan-based chemical building blocks from sustainable feedstocks.

Keywords: 5-hydroxymethylfurfural, 2,5-diformylfuran, galactose oxidase, biocatalysis, directed evolution

Introduction

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[1]. HMF can be transformed through different reactions to produce a range of derivatives, many having applications in the polymer industry[2-4]. In particular, the oxidized HMF derivatives 2,5-diformylfuran (DFF) and furan-2,5-dicarboxylic acid (FDCA) are both important intermediates in furan based polymer synthesis (Figure 1), as they can be condensed with other monomers to make poly-imines, -esters, -amides and -urethanes as plastics, resins and porous organic frameworks[2,5,6]. In addition to more environmentally

responsible manufacturing methods[7], 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[8,9], and some even display new properties of potential use in performance materials[10,11].

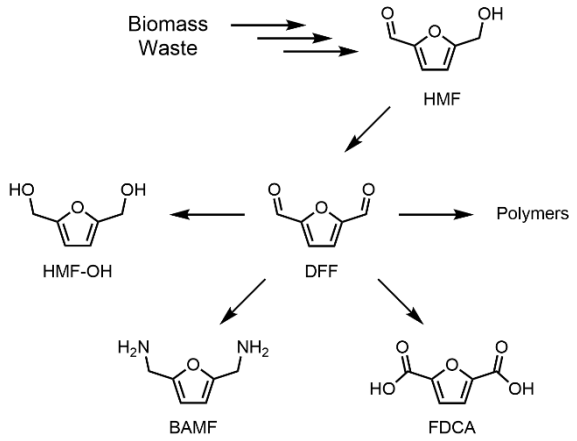

Figure 1. Diversification of HMF to high value products via DFF intermediate.

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[4,12-17]. 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[4,14,18]. Once in hand, HMF can be oxidized by a variety of metal catalysts or electrochemical methods to produce DFF, FDCA or its dimethyl ester (FDME) which are more stable monomers[2,4,19,20]. 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[21,22], primarily chloroperoxidases[23], HMF oxidases (HMFO)[24], aryl-alcohol oxidases[25,26] and galactose oxidases (GOase) as well as related copper radical oxidases[27-29]. The latter three seem to be the most appealing due to their dependence on oxygen as a co-substrate rather than H_2O_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[30-34]. HMF oxidases and aryl-alcohol oxidases are also interesting in that they catalyse the complete oxidation of HMF to FDCA[24-26,35-37]. In analogous two-enzyme systems, GOase has been combined with periplasmic aldehyde oxidase PaoABC[28] or unspecific peroxygenase[38,39] 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. In fact, DFF has been particularly challenging to selectively synthesize efficiently (chemically or biocatalytically) without requiring high levels of (bio)catalyst[22,40,41].

Building on our previous work in understanding the effect of reaction conditions on GOase[42], 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 DFF. Parallel process 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 process driven, integrated approach comprising both process and enzyme engineering[43,44] has resulted in an effective and high yielding model process for the selective 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 process metric targets to guide our approach based on estimated threshold values for a biocatalytic processes in the bulk chemical sector[45,46] (Table 1). These metrics were then used to benchmark the industrial feasibility of DFF production and help define which aspects were still in need of improvement.

Table 1. Target process metrics for oxidation of HMF to DFF by GOase.

	Unit	Target
Final Prod. Conc.	g/L	100
Duration	h	25
Productivity	g/L.h	4
Specific Yield[a]	$g_{Product}/g_{Biocatalyst}$ [b]	1000
Conversion	%	98
Isolated Yield	%	98
Purity	%	>98

[a]Specific yield is defined as (mass product over the biocatalyst lifetime)/mass biocatalyst.
[b]$g_{Biocatalyst}$ taken as pure enzyme or pure enzyme equivalent in CFE based on specific activity.

Our approach began with enzyme engineering that was guided by reaction constraints to directly develop a biocatalyst that was industrially relevant, and was balanced by concurrent, iterative process development to tune conditions and push performance limits to identify scalable 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[44,47]. In the interests of clarity, work on these approaches is discussed in two separate subsections below, despite being integrated in reality.

Enzyme Engineering

The first round of library generation targeted pairs of active site residues (Figure 2), and screening against 1-hexanol identified variant M_4 (M_{3-5} + 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_4. Mutated sites in variants published by Delagrave *et al.* (variant 7.5.1 with

C383S/Y436H/N318D/V477D/A626S/V494A)[48], Wilkinson *et al.* (variant C383S/Y436H)[49], and Deacon and McPherson (C383S and C383T)[50] were chosen as sites for potential non-selective catalytic improvements. None of these residues interact directly with the substrate (Supplementary Information, Figure S1), 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 Information, Table S1), were randomly introduced to create a library of new combinations and screened for activity on 1-hexanol. The top hits identified from this screen were variants M_{5-1} (V477D/A626S), and M_{5-2} (N318D/C383T/Y436H/V477D). Interestingly, these hits had unique sequences compared to the previously published variants. Kinetic characterization of the new 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.

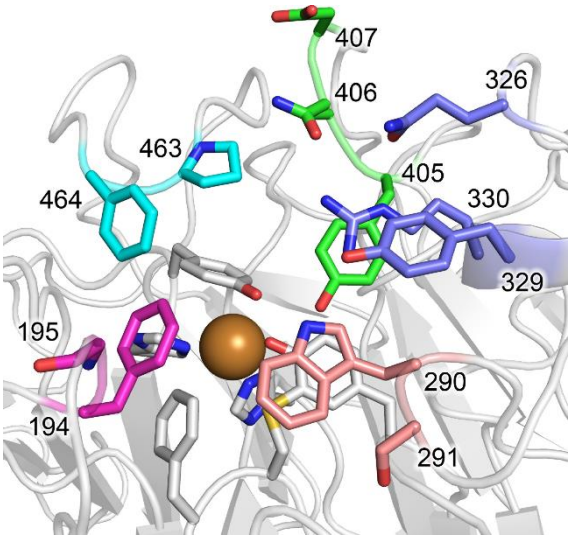

Figure 2. Positions of active site residues targeted in the seven GOase M_{3-5} NNK CASTing libraries, displayed on wildtype GOase crystal structure (PDBID: 1GOG). Libraries were A: 406/407, B: 326/330, C: 290/291, D: 194/195, E: 463/464, F: 405/406 and G: 329/330.

Table 2. Apparent kinetic parameters for GOase variants with HMF.

Variant Name	$k_{cat,app}$ (s^{-1})	$K_{M,app}$ (mM)	k_{cat}/K_M (s^{-1} M^{-1})
M_{3-5}	123.9 ± 1.4	3.3 ± 0.1	38000 ± 1000
M_4	132.7 ± 2.6	2.1 ± 0.2	63000 ± 6000
M_{5-1}	153.9 ± 4.2	2.0 ± 0.2	77000 ± 8000
M_{5-2}	139.0 ± 2.4	1.8 ± 0.1	77000 ± 4000
M_{6-A}	218.5 ± 14.6	12.9 ± 2.7	17000 ± 4000
M_{6-B}	121.7 ± 6.9	17.7 ± 2.9	6900 ± 1200
M_{7-1A}	246.2 ± 10.7	15.3 ± 2.2	16000 ± 2000
M_{7-2A}	204.6 ± 10.7	14.5 ± 2.6	14000 ± 3000

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[33,51]. Oxygen has a low solubility in aqueous solution (~270 µM)[31,33,52], while

K_M for O_2 (K_{MO}) for many enzymes is often estimated to be much higher than the oxygen concentration in equilibrium with air[33,53]. This difference means that reaction rates will be dependent upon the oxygen concentration in the liquid and thereby on oxygen transfer rates[42]. This limitation is magnified at high substrate loadings, where substrate concentration and oxygen concentration can differ by a factor of up to 10^3 [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_{MO} of the biocatalyst, or in other words, engineer the enzyme to work more effectively at lower oxygen concentrations[42,54-57]. Likewise, improvements in k_{cat}/K_{MO} (catalytic efficiency for oxygen) will be more beneficial than improvements in k_{cat}/K_{MS} (where K_{MS} 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 (Figure 2) on the GOase M_4 template were screened for improved GOase activity at low oxygen levels using HMF as substrate. Despite the extremely low oxygen concentration, multiple potential hits from libraries C and D were identified. Upon confirmation in a secondary screen and basic characterization, GOase $M_{6\text{-}A}$ (F290W/S291S) was identified as the top performing variant. Additionally, one potential hit from library C (variant $M_{6\text{-}B}$, 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[58], but was mutated to phenylalanine during engineering of the GOase M_3 variant[59] (Supplementary Information, Table S1). It appears that W290 has a significant impact on substrate (HMF and/or O_2) binding and catalysis since the $k_{cat,app}$ for HMF in the $M_{6\text{-}A}$ variant increased by 65%, while the $K_{M,app}$ increased by approximately 6-fold (Table 2). The $K_{M,app}$ for $M_{6\text{-}B}$ was similarly affected, although there was little change in $k_{cat,app}$ 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 $M_{7\text{-}1A}$, $M_{7\text{-}2A}$, $M_{7\text{-}1B}$ and $M_{7\text{-}2B}$ variants (Supplementary Information, Table S1). $M_{7\text{-}1A}$ and $M_{7\text{-}2A}$ were kinetically characterized, revealing an increase in $k_{cat,app}$ to 246 s^{-1} for $M_{7\text{-}1A}$ (Table 2), which compares quite favourably to FAD-dependent enzymes that oxidize HMF[24-26,36,37], and is similar to another copper radical oxidase variant[27]. In this instance, the combination of mutations from $M_{5\text{-}1}$ and $M_{5\text{-}2}$ with $M_{6\text{-}A}$ 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)[60], which has enabled a simplified method for accurately determining true kinetic parameters for oxygen-dependent enzymes, specifically K_{MO}[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_1) variant: the initial $M_{3\text{-}5}$ progenitor, the first hit for improved HMF activity (M_4) and the top hit from the low O_2 screen ($M_{6\text{-}A}$).

K_{MO} 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_{MO} can be observed by comparison of the TiTR data with the apparent kinetic parameters reported in Table 2. Measurements for the M_4 and $M_{6\text{-}A}$ variants are fairly consistent between the two experiments as a result of K_{MO} values near or below the level of aqueous O_2 concentration in equilibrium with air (~270 µM and 30°C). However, the high K_{MO} for GOase $M_{3\text{-}5}$ shows that this variant was severely oxygen limited in the standard kinetic assay and was therefore much more responsive to the increase in aqueous O_2 concentration in the TiTR. The large improvement in k_{cat}/K_{MO} found in the $M_{6\text{-}A}$ variant as a result of the low oxygen assay conditions highlights the value of using this screen for engineering oxygen dependent enzymes, and confirms that it is indeed possible to intentionally target and improve reactivity in GOase. Furthermore, we believe that this glovebox modification could be generally applied to provide a unique selective pressure for engineering K_{MO} in other oxygen-dependent enzymes, which could significantly accelerate their development for use at industrial scale.

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

Variant	K_{MO} (mM)	K_{MS} (mM)	k_{cat} (s^{-1})	k_{cat}/K_{MO} (M^{-1} s^{-1})	k_{cat}/K_{MS} (M^{-1} s^{-1})
M_1	0.16 ± 0.11	53 ± 18	23.5 ± 4.7	(1.5 ± 1.1)•10^5	(0.04 ± 0.02)•10^4
$M_{3\text{-}5}$[a]	1.39 ± 0.46	14.9 ± 4.5	651 ± 132	(4.7 ± 1.8)•10^5	(4.4 ± 1.6)•10^4
M_4	0.37 ± 0.07	1.83 ± 0.30	120 ± 6	(3.3 ± 0.6)•10^5	(6.6 ± 1.1)•10^4
$M_{6\text{-}A}$	0.15 ± 0.02	6.19 ± 0.41	166 ± 4	(10.9 ± 1.5)•10^5	(2.7 ± 0.2)•10^4

K_{MO}: K_M for oxygen, K_{MS}: K_M for HMF. [a]Measured using CFE powder with an estimated GOase content, giving a higher uncertainty in the k_{cat} parameter.

Process Engineering

Process engineering for the GOase catalysed oxidation of HMF to DFF was performed alongside the enzyme engineering work, incorporating newly improved variants as they became available. Initial reaction compositions were based on our previously published work[42] and were then adapted in stages. As described below, this approach allowed an assessment of the limits for a given mutant while also considering scalability.

Initial reactions to characterise over-oxidation of DFF to 5-formyl-2-furan carboxylic acid (FFCA) by GOase variants[61] indicated that this was not a concern, and identified M_4 as the more effective biocatalyst for HMF oxidation (Supplementary Note and Supplementary Table S2). From there, we next started a process of reaction optimization beginning with screening for compatible co-solvents. An initial screen of typical solvents highlighted DMSO as a beneficial and compatible solvent, and 0.05 g/L (0.71 µM) purified GOase as an ideal working biocatalyst loading giving high conversion of 100 mM HMF and good conversion of 250 mM HMF (Supplementary Note and Supplementary Information, Tables S3 and S4). Additionally, GOase M_4 performed well when challenged with a preparation of crude HMF from BASF (Supplementary Information, Table S5). An important consideration in the accessibility of furan-based materials from biomass is the processing necessary to convert the crude HMF into a formulation suitable for the subsequent reaction. As mentioned, HMF is frequently required in purified form before it can be converted satisfactorily by many inorganic catalysts, thus the option to overcome such purification requirements by using crude HMF could lead to significantly reduced production costs. Similarly, use of a crude biocatalyst formulation such as dried cell free extract could prove more cost effective. In the first instance, an approximately

equivalent GOase M_4 loading of dried CFE formulation (5-6% GOase by weight) gave slightly higher conversions than the purified enzyme (Supplementary Information, Table S6).

Attempting to remove DMSO to avoid future process complications resulted in reduced productivity (comparing Supplementary Information, Table S4 Entry 3 and, Table S6 Entry 1). To find a more suitable solvent for scale-up, we then examined M_4 performance in a variety of water-miscible and -immiscible co-solvents (Supplementary Information, Table S7 and S8), in addition to the effect of reaction temperature (Supplementary Information, Table S9). The most promising results were observed when using ethyl acetate (EtOAc) at 20°C. It is expected that the addition of a water-immiscible organic solvent, as well as the lower temperature, both contribute to improved stability by reducing the aqueous concentration of the dialdehyde product. Dialdehydes are common crosslinking reagents[62] and can modify accessible lysine residues (22 in the case of this construct), often reducing enzyme activity and stability. Accordingly, effective removal of the DFF product should enable higher conversions.

Integrating these new 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 M_4 as purified enzyme and as CFE after 6 h, suggesting that substrate loading could be pushed still higher (Table 4 and Supplementary Information, Table S10). Indeed, under the same conditions, conversion to DFF reached 70% of 500 mM HMF and 81% of 50 g/L crude HMF by M_4 CFE after 6 h.

Table 4. Conversion of HMF to DFF by GOase variant M_4 CFE using EtOAc overlay at reduced incubation temperature. Data for reactions with purified enzyme provided in Supplementary Information, Table S10.

Entry	GOase Variant	GOase (g/L)	GOase Form	HRP (g/L)	[HMF]	Type HMF	Conversion (%)	Over-oxidation (%)
1	**M_4**	0.625	CFE	0.0128	250 mM	Pure	97	2.5
2	**M_4**	0.625	CFE	0.0128	25 g/L	Crude	89	6.5
3	**M_4**	0.625	CFE	0.0128	500 mM	Pure	70	0.4
4	**M_4**	0.625	CFE	0.0128	50 g/L	Crude	81	0.8

Conditions: 0.05 mM $CuSO_4$ in GOase M_4 CFE reactions, 880 U/mL Catalase, 100 mM NaPi, 200 µL EtOAc, 20°C, 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 Figure S2). Co-solvents that proved to be more beneficial and compatible with GOase were esters, carbonates and ethers, with diethyl carbonate (DEC, bp 126°) and butyl acetate (BuOAc, 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 HMF using purified GOase M_4, and also at 50 g/L crude HMF when using GOase M_4 as CFE (Table 5 and Supplementary Information, Table S11). This high level of conversion of crude HMF by the unpurified GOase preparation as CFE (Table 5, Entry 2) now approaches industrially relevant metrics (Table 1), while utilizing a biocatalyst formulation that is more realistic for industrial application. Working at these high substrate loadings, we suspected that the benefits of the biphasic reaction were two-fold. In effect, the second liquid phase was

acting as a reservoir both for HMF as well as DFF, continuously feeding the substrate while extracting the product. These expectations are generally supported by the experimentally determined partition of HMF and DFF between the aqueous and organic layers (Supplementary Information, Table S12).

Table 5. Conversion of HMF to DFF by GOase variant M_4 CFE at high HMF loading in the presence of different cosolvents. Data for reactions with purified enzyme provided in Supplementary Information, Table S11.

Entry	GOase Variant	GOase (g/L)	GOase Form	HRP (g/L)	Co-Solvent	[HMF] (g/L)	Type HMF	Conversion (%)	Over-oxidation (%)
1	**M_4**	0.625	CFE	0.0128	EtOAc	50	Crude	81	0.8
2	**M_4**	0.625	CFE	0.0128	DEC	50	Crude	80	0.8
3	**M_4**	0.625	CFE	0.0128	BuOAc	50	Crude	73	0.7

Conditions: 0.05 mM $CuSO_4$ in GOase M_4 CFE reactions, 880 U/mL Catalase, 100 mM NaPi, 200 µL solvent overlay, 20°C, 6 h.

The four newly identified Round 2 GOase variants displayed greatly improved conversion of 50 g/L semi-crude HMF compared to the M_4 variant (Supplementary Note, Supplementary Information, Table S13), with the best variant ($M_{6\text{-}A}$) reaching nearly 90% conversion after 6 h. This was particularly significant since the variants were engineered in two distinctly different screening strategies: in one case targeting distal residues for increased k_{cat}, and in the other targeting active site residues for lower $K_{MO.}$ The combination of these mutations in Round 3 variants $M_{7\text{-}1A}$, $M_{7\text{-}2A}$, $M_{7\text{-}1B}$ and $M_{7\text{-}2B}$ led to an additive effect and still greater conversion than the parental variants (up to 79%) when tested at 100 g/L semi-crude HMF (Table 6). All variants holding the unique $M_{6\text{-}A}$ 'A' mutations (F290W/S291S) had a faster rate of conversion and high overall conversion, which reflects the increased $k_{cat,app}$ values measured for these variants (Table 2). Additionally, variants having the unique $M_{6\text{-}B}$ 'B' mutations (F290W/S291R), were the only variants that showed a significant increase in conversion after the 6 h time point, indicating that the additional S291R does indeed have a beneficial effect on stability, potentially via formation of a salt bridge with E195. However, the S291R mutation appears to prevent a similar improvement in turnover rate provided by F290W in the $M_{6\text{-}A}$ variant.

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	**M_4**	28	28
3/4	**$M_{5\text{-}1}$**	36	36
5/6	**$M_{5\text{-}2}$**	49	51
7/8	**$M_{6\text{-}A}$**	54	59
9/10	**$M_{6\text{-}B}$**	56	74
11/12	**$M_{7\text{-}1A}$**	61	66
13/14	**$M_{7\text{-}2A}$**	76	75
15/16	**$M_{7\text{-}1B}$**	61	79
17/18	**$M_{7\text{-}2B}$**	55	77

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), 20°C, 6 and 24 h. Over-oxidation in all samples was found to be <0.3%.

With an interest to identify a broader range of solvents that could potentially improve downstream processing of the reaction, several other co-solvents that offered slightly different

physical properties (density, Log P, water solubility, boiling point, etc., Supplementary Information, Table S14) 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 Information, Figure S3). These four solvents (DEC, DEM, DES and EGDA) were then used to evaluate the top performing GOase variants ($M_{6\text{-}B}$, $M_{7\text{-}2A}$, $M_{7\text{-}1B}$ and $M_{7\text{-}2B}$) challenged with 150 g/L (~1.2 M) semi-crude HMF (Figure 3). Despite the apparent stability improvements of the 'B' variants, GOase $M_{7\text{-}2A}$ was consistently the most productive in all reaction compositions, reaching up to 64% conversion after 24 h with DEM as the co-solvent, remarkably approaching 100 g/L product concentration.

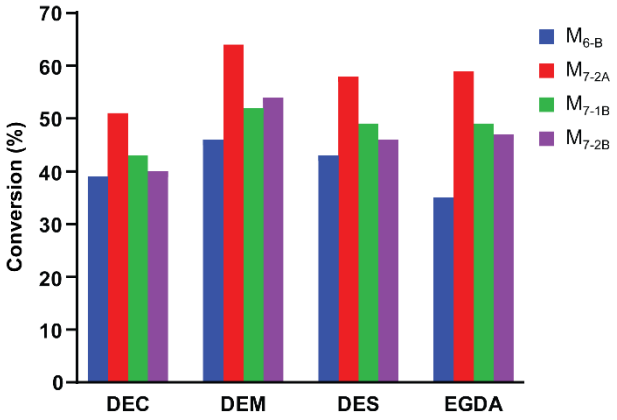

Figure 3. Conversion of 150 g/L semi-crude HMF to DFF by GOase variants in the presence of different cosolvents. Conditions: 0.05 g/L pure enzyme, 0.0064 g/L HRP, 880 U/mL Catalase, 100 mM NaPi, 150 g/L Crude HMF in DEC (BASF), 20°C, 24 h. No over-oxidation was observed in any of the samples. Abbreviations: DEC, diethyl carbonate. DEM, diethyl malonate. DES, diethyl succinate. EGDA, ethylene glycol diacetate.

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 1.07×10^6 for the GOase $M_{7\text{-}2A}$variant in DEM, giving an excellent comparison to TTN ranges for other industrially applicable biocatalysts[63-67]. The highest productivity though was achieved with GOase $M_{7\text{-}2A}$ at 100 g/L semi-crude HMF in DEC after 6 h (Table 6 Entry 13), reaching 12.5 g/L/h (aqueous volume only, or 6.9 g/L/h when including solvent volume) and a specific yield 1,500 g_{DFF}/g_{enzyme}. This represents a nine-fold increase in productivity with a seven-fold increase in specific yield when compared to the $M_{3\text{-}5}$ progenitor enzyme at 1.4 g/L/h and 218 g_{DFF}/g_{enzyme} (Supplementary Information, Table S2, 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 $M_{7\text{-}2A}$. After 6 h, 100 g/L HMF was almost fully converted to DFF (96%), while conversion of 150 g/L loading lagged behind (59%) (Supplementary Information, Table S15). 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 process work provided a benchmark against which to measure reactions at larger scale (Supplementary

Information, Table S16). GOase variants $M_{3\text{-}5}$, $M_{6\text{-}A}$ and $M_{7\text{-}2A}$ were selected to highlight the improvements in performance gained through the low oxygen screening conditions ($M_{6\text{-}A}$) and recombined best mutations ($M_{7\text{-}2A}$) compared to the initial progenitor ($M_{3\text{-}5}$).

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 $M_{3\text{-}5}$ reached 24% conversion of 50 g/L HMF, while GOase $M_{6\text{-}A}$ and $M_{7\text{-}2A}$ had similarly improved performance at 37% and 36% conversion, respectively (Supplementary Information, Figure S4a and Supplementary Information, Table S17, Entries 1-3). This conversion was lower than expected from the analytical scale reactions, which we surmised was 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 H_2O_2 [42]. Indeed, when the catalase concentration was increased in reactions using $M_{7\text{-}2A}$, up to 59% conversion was observed after 6 h (Supplementary Information, Figure S4b and Supplementary Information, Table S17, Entries 4-5) indicating that catalase activity was a limiting factor. These reactions, however, also exhibited some foaming. 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 30.6 $g_{DFF}/g_{biocatalyst}$ as CFE, or 569 $g_{DFF}/g_{biocatalyst}$ as pure enzyme equivalent (Supplementary Information, Table S17). 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 $g_{DFF}/g_{biocatalyst}$), demonstrating the feasibility of these reaction conditions and process design.

Conclusions

The results presented here highlight the value of integrating informed reaction and process considerations into the enzyme engineering workflow to directly develop a biocatalyst that balances process and enzyme requirements. Using this integrated approach to process and enzyme engineering, we have developed an advanced GOase variant and corresponding process conditions for effective production of the bio-based chemical building block DFF that could be used directly in further enzymatic (or chemo-catalytic) transformations[28,68,69] to produce other key bio-derived furans for industrial applications. The low catalyst loading of our GOase $M_{7\text{-}2A}$ variant combined with the achievement of high conversion and TTN at >1 M substrate concentrations is unique for these biocatalysts 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 process conditions represent the most efficient and selective biocatalytic production of DFF to date, and certainly compares favourably with available chemo-catalytic synthetic methods[22,40,41].

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 higher substrate loadings and production titres to meet expected production scale demands. These could potentially be found through additional enzyme engineering to improve aldehyde tolerance, 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. Furthermore, methods for bulk biocatalyst production in an expression host need to be re-validated for this new GOase variant, presumably based on the well-established *Pichia* expression systems for GOase[71]. 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. Finally, identifying a continuously available carbohydrate feedstock to supply an inexpensive and reliable method for HMF synthesis[4,12-17] will be critically important to allow uninterrupted production of DFF for use in industry. Although these obstacles still stand in the way of commercial production of DFF, each has significant precedent to be achievable and the largest truly missing piece was a highly productive (bio)catalyst for HMF oxidation. With this biocatalyst now in hand, broader production and use of these bio-based furan polymers is closer to being in sight.

Associated Content

Supplementary Information

The supporting information is available at (DOI once available).

Author Information

Corresponding Author:

*nicholas.turner@manchester.ac.uk.

Notes:

The authors declare no competing financial interests.

Acknowledgements

The research leading to these results has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 613849 supporting the project BIOOX. The authors would also like to thank James Marshall (University of Manchester) and Darren Cook and Simon Charnock (Prozomix Ltd.) for assistance in CFE production, as well as Derren Heyes (University of Manchester) for access to equipment to perform the low oxygen screening assay.

References

1 Bozell, J. J. & Petersen, G. R. Technology development for the production of biobased products from biorefinery carbohydrates - The US Department of Energy's "Top 10" revisited. *Green Chem.* **12**, 539-554, (2010).

2 Delidovich, I. *et al.* Alternative monomers based on lignocellulose and their use for polymer production. *Chem. Rev.* **116**, 1540-1599, (2016).

3 Sousa, A. F. *et al.* Biobased polyesters and other polymers from 2,5-furandicarboxylic acid: a tribute to furan excellency. *Polym. Chem.* **6**, 5961-5983, (2015).

4 Teong, S. P., Yi, G. S. & Zhang, Y. G. Hydroxymethylfurfural production from bioresources: Past, present and future. *Green Chem.* **16**, 2015-2026, (2014).

5 Amarasekara, A. S., Green, D. & Williams, L. D. Renewable resources based polymers: Synthesis and characterization of 2,5-diformylfuran-urea resin. *Eur. Polym. J.* **45**, 595-598, (2009).

6 Ma, J. *et al.* Synthesis and properties of furan-based imine-linked porous organic frameworks. *Polym. Chem.* **3**, 2346-2349, (2012).

7 Eerhart, A., Faaij, A. P. C. & Patel, M. K. Replacing fossil based PET with biobased PEF; process analysis, energy and GHG balance. *Energy Environ. Sci.* **5**, 6407-6422, (2012).

8 de Jong, E., Dam, M. A., Sipos, L. & Gruter, G. J. M. in *Biobased Monomers, Polymers, and Materials* Vol. 1105 *ACS Symposium Series* (eds P. B. Smith & R. A. Gross) 1-13 (2012).

9 Rosenboom, J.-G., Hohl, D. K., Fleckenstein, P., Storti, G. & Morbidelli, M. Bottle-grade polyethylene furanoate from ring-opening polymerisation of cyclic oligomers. *Nat. Commun.* **9**, 2701, (2018).

10 Hui, Z. & Gandini, A. Polymeric Schiff bases bearing furan moieties. *Eur. Polym. J.* **28**, 1461-1469, (1992).

11 Liu, C.-L. *et al.* Theoretical analysis on the geometries and electronic structures of coplanar conjugated poly(azomethine)s. *Polymer* **46**, 4950-4957, (2005).

12 Li, M. *et al.* High conversion of glucose to 5-hydroxymethylfurfural using hydrochloric acid as a catalyst and sodium chloride as a promoter in a water/γ-valerolactone system. *RSC Adv.* **7**, 14330-14336, (2017).

13 Mika, L. T., Csefalvay, E. & Nemeth, A. Catalytic conversion of carbohydrates to initial platform chemicals: Chemistry and sustainability. *Chem. Rev.* **118**, 505-613, (2018).

14 Motagamwala, A. H. *et al.* Toward biomass-derived renewable plastics: Production of 2,5-furandicarboxylic acid from fructose. *Sci. Adv.* **4**, (2018).

15 Pyo, S.-H., Sayed, M. & Hatti-Kaul, R. Batch and continuous flow production of 5-hydroxymethylfurfural from a high concentration of fructose using an acidic ion exchange catalyst. *Org. Process Res. Dev.* **23**, 952-960, (2019).

16 Roman-Leshkov, Y., Chheda, J. N. & Dumesic, J. A. Phase modifiers promote efficient production of hydroxymethylfurfural from fructose. *Science* **312**, 1933-1937, (2006).

17 Toftgaard Pedersen, A., Ringborg, R., Grotkjær, T., Pedersen, S. & Woodley, J. M. Synthesis of 5-hydroxymethylfurfural (HMF) by acid catalyzed dehydration of glucose–fructose mixtures. *Chem. Eng. J.* **273**, 455-464, (2015).

18 Gallo, J. M. R., Alonso, D. M., Mellmer, M. A. & Dumesic, J. A. Production and upgrading of 5-hydroxymethylfurfural using heterogeneous catalysts and biomass-derived solvents. *Green Chem.* **15**, 85-90, (2013).

19 Sajid, M., Zhao, X. & Liu, D. Production of 2,5-furandicarboxylic acid (FDCA) from 5-hydroxymethylfurfural (HMF): recent progress focusing on the chemical-catalytic routes. *Green Chem.* **20**, 5427-5453, (2018).

20 Zhang, Z. & Deng, K. Recent advances in the catalytic synthesis of 2,5-furandicarboxylic acid and its derivatives. *ACS Catal.* **5**, 6529-6544, (2015).

21 Cajnko, M. M., Novak, U., Grilc, M. & Likozar, B. Enzymatic conversion reactions of 5-hydroxymethylfurfural (HMF) to bio-based 2,5-diformylfuran (DFF) and 2,5-furandicarboxylic acid (FDCA) with air: mechanisms, pathways and synthesis selectivity. *Biotecnhol. Biofuels* **13**, 66, (2020).

22 Hu, L. *et al.* Biocatalytic transformation of 5-hydroxymethylfurfural into high-value derivatives: Recent advances and future aspects. *ACS Sustain. Chem. Eng.* **6**, 15915-15935, (2018).

23 van Deurzen, M. P. J., van Rantwijk, F. & Sheldon, R. A. Chloroperoxidase-catalyzed oxidation of 5-hydroxymethylfurfural. *J. Carbohydr. Chem.* **16**, 299-309, (2006).

24 Dijkman, W. P. & Fraaije, M. W. Discovery and characterization of a 5-hydroxymethylfurfural oxidase from Methylovorus sp. strain MP688. *Appl. Environ. Microbiol.* **80**, 1082-1090, (2014).

25 Carro, J. *et al.* 5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. *FEBS J.* **282**, 3218-3229, (2015).

26 Vina-Gonzalez, J., Martinez, A. T., Guallar, V. & Alcalde, M. Sequential oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid by an evolved aryl-alcohol oxidase. *BBA-Proteins Proteom* **1868**, 140293, (2020).

27 Mathieu, Y. *et al.* Discovery of a fungal copper radical oxidase with high catalytic efficiency toward 5-hydroxymethylfurfural and benzyl alcohols for bioprocessing. *ACS Catal.* **10**, 3042-3058, (2020).

28 McKenna, S. M., Leimkühler, S., Herter, S., Turner, N. J. & Carnell, A. J. Enzyme cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem. *Green Chem.* **17**, 3271-3275, (2015).

29 Qin, Y.-Z., Li, Y.-M., Zong, M.-H., Wu, H. & Li, N. Enzyme-catalyzed selective oxidation of 5-hydroxymethylfurfural (HMF) and separation of HMF and 2,5-diformylfuran using deep eutectic solvents. *Green Chem.* **17**, 3718-3722, (2015).

30 Baldwin, C. V. & Woodley, J. M. On oxygen limitation in a whole cell biocatalytic Baeyer-Villiger oxidation process. *Biotechnol. Bioeng.* **95**, 362-369, (2006).

31 Chapman, M. R., Cosgrove, S. C., Turner, N. J., Kapur, N. & Blacker, A. J. Highly productive oxidative biocatalysis in continuous flow by enhancing the aqueous equilibrium solubility of oxygen. *Angew. Chem. Int. Ed. Engl.* **57**, 10535-10539, (2018).

32 Hoschek, A., Buhler, B. & Schmid, A. Overcoming the gas-liquid mass transfer of oxygen by coupling photosynthetic water oxidation with biocatalytic oxyfunctionalization. *Angew. Chem. Int. Ed. Engl.* **56**, 15146-15149, (2017).

33 Ringborg, R. H., Toftgaard Pedersen, A. & Woodley, J. M. Automated determination of oxygen-dependent enzyme kinetics in a tube-in-tube flow reactor. *ChemCatChem* **9**, 3285-3288, (2017).

34 Toftgaard Pedersen, A. *et al.* Characterization of a continuous agitated cell reactor for oxygen dependent biocatalysis. *Biotechnol. Bioeng.* **114**, 1222-1230, (2017).

35 Dijkman, W. P., Binda, C., Fraaije, M. W. & Mattevi, A. Structure-based enzyme tailoring of 5-hydroxymethylfurfural oxidase. *ACS Catal.* **5**, 1833-1839, (2015).

36 Dijkman, W. P., Groothuis, D. E. & Fraaije, M. W. Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid. *Angew. Chem. Int. Ed. Engl.* **53**, 6515-6518, (2014).

37 Martin, C., Maqueo, A. O., Wijma, H. J. & Fraaije, M. W. Creating a more robust 5-hydroxymethylfurfural oxidase by combining computational predictions with a novel effective library design. *Biotecnhol. Biofuels* **11**, (2018).

38 Carro, J. *et al.* Self-sustained enzymatic cascade for the production of 2,5-furandicarboxylic acid from 5-methoxymethylfurfural. *Biotecnhol. Biofuels* **11**, (2018).

39 Kalum, L. *et al.* Enzymatic oxidation of 5-hydroxymethylfurfural and derivatives thereof. WO 2014/015256 A2 (2014).

40 Kucherov, F. A., Romashov, L. V., Galkin, K. I. & Ananikov, V. P. Chemical transformations of biomass-derived C6-furanic platform chemicals for sustainable energy research, materials science, and synthetic building blocks. *ACS Sustain. Chem. Eng.* **6**, 8064-8092, (2018).

41 Zhang, Z. H. & Huber, G. W. Catalytic oxidation of carbohydrates into organic acids and furan chemicals. *Chem. Soc. Rev.* **47**, 1351-1390, (2018).

42 Toftgaard Pedersen, A. *et al.* Process requirements of galactose oxidase catalyzed oxidation of alcohols. *Org. Process Res. Dev.* **19**, 1580-1589, (2015).

43 Woodley, J. M. Protein engineering of enzymes for process applications. *Curr. Opin. Chem. Biol.* **17**, 310-316, (2013).

44 Woodley, J. M. Integrating protein engineering with process design for biocatalysis. *Philos. Trans. R. Soc. A* **376**, (2017).

45 Tufvesson, P., Lima-Ramos, J., Haque, N. A., Gernaey, K. V. & Woodley, J. M. Advances in the process development of biocatalytic processes. *Org. Process Res. Dev.* **17**, 1233-1238, (2013).

46 Lima-Ramos, J., Tufvesson, P. & Woodley, J. M. Application of environmental and economic metrics to guide the development of biocatalytic processes. *Green Processing and Synthesis* **3**, 195-213, (2014).

47 Lima-Ramos, J., Neto, W. & Woodley, J. M. Engineering of biocatalysts and biocatalytic processes. *Top. Catal.* **57**, 301-320, (2014).

48 Delagrave, S. *et al.* Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase. *Protein Eng.* **14**, 261-267, (2001).

49 Wilkinson, D. *et al.* Structural and kinetic studies of a series of mutants of galactose oxidase identified by directed evolution. *Protein Eng. Des. Sel.* **17**, 141-148, (2004).

50 Deacon, S. E. & McPherson, M. J. Enhanced expression and purification of fungal galactose oxidase in Escherichia coli and use for analysis of a saturation mutagenesis library. *ChemBioChem* **12**, 593-601, (2011).

51 Bommarius, A. S. & Karau, A. Deactivation of formate dehydrogenase (FDH) in solution and at gas-liquid interfaces. *Biotechnol. Progr.* **21**, 1663-1672, (2005).

52 Wilhelm, E., Battino, R. & Wilcock, R. J. Low-pressure solubility of gases in liquid water. *Chem. Rev.* **77**, 219-262, (1977).

53 Bar-Even, A. *et al.* The moderately efficient enzyme: Evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* **50**, 4402-4410, (2011).

54 Baron, R. *et al.* Multiple pathways guide oxygen diffusion into flavoenzyme active sites. *Proc. Natl. Acad. Sci.* **106**, 10603-10608, (2009).

55 Piubelli, L. *et al.* On the oxygen reactivity of flavoprotein oxidases: An oxygen access tunnel and gate in Brevibacterium sterolicum cholesterol oxidase. *J. Biol. Chem.* **283**, 24738-24747, (2008).

56 Rosini, E., Molla, G., Ghisla, S. & Pollegioni, L. On the reaction of D-amino acid oxidase with dioxygen: O2 diffusion pathways and enhancement of reactivity. *FEBS J.* **278**, 482-492, (2011).

57 Rosini, E., Pollegioni, L., Ghisla, S., Orru, R. & Molla, G. Optimization of D-amino acid oxidase for low substrate concentrations - towards a cancer enzyme therapy. *FEBS J.* **276**, 4921-4932, (2009).

58 Sun, L. H., Petrounia, I. P., Yagasaki, M., Bandara, G. & Arnold, F. H. Expression and stabilization of galactose oxidase in Escherichia coli by directed evolution. *Protein Eng.* **14**, 699-704, (2001).

59 Sun, L. H., Bulter, T., Alcalde, M., Petrounia, I. P. & Arnold, F. H. Modification of galactose oxidase to introduce glucose 6-oxidase activity. *ChemBioChem* **3**, 781-783, (2002).

60 Polyzos, A., O'Brien, M., Petersen, T. P., Baxendale, I. R. & Ley, S. V. The continuous-flow synthesis of carboxylic acids using CO2 in a tube-in-tube gas permeable membrane reactor. *Angew. Chem. Int. Ed. Engl.* **50**, 1190-1193, (2011).

61 Birmingham, W. R. & Turner, N. J. A single enzyme oxidative “cascade” via a dual-functional galactose oxidase. *ACS Catal.* **8**, 4025-4032, (2018).

62 Migneault, I., Dartiguenave, C., Bertrand, M. J. & Waldron, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques* **37**, 790-802, (2004).

63 Bommarius, A. S. & Paye, M. F. Stabilizing biocatalysts. *Chem. Soc. Rev.* **42**, 6534-6565, (2013).

64 Bosshart, A., Wagner, N., Lei, L., Panke, S. & Bechtold, M. Highly efficient production of rare sugars D-psicose and L-tagatose by two engineered D-tagatose epimerases. *Biotechnol. Bioeng.* **113**, 349-358, (2016).
65 de Almeida, T. P. *et al.* Efficient Aerobic oxidation of trans-2-hexen-1-ol using the aryl alcohol oxidase from Pleurotus eryngii. *Adv. Synth. Catal.* **361**, 2668-2672, (2019).
66 Rogers, T. A. & Bommarius, A. S. Utilizing simple biochemical measurements to predict lifetime output of biocatalysts in continuous isothermal processes. *Chem. Eng. Sci.* **65**, 2118-2124, (2010).
67 Seelbach, K., vanDeurzen, M. P. J., vanRantwijk, F., Sheldon, R. A. & Kragl, U. Improvement of the total turnover number and space-time yield for chloroperoxidase catalyzed oxidation. *Biotechnol. Bioeng.* **55**, 283-288, (1997).
68 Dunbabin, A., Subrizi, F., Ward, J. M., Sheppard, T. D. & Hailes, H. C. Furfurylamines from biomass: transaminase catalysed upgrading of furfurals. *Green Chem.* **19**, 397-404, (2017).
69 McKenna, S. M. *et al.* The continuous oxidation of HMF to FDCA and the immobilisation and stabilisation of periplasmic aldehyde oxidase (PaoABC). *Green Chem.* **19**, 4660-4665, (2017).
70 Hulsewede, D., Meyer, L. E. & von Langermann, J. Application of in situ product crystallization and related techniques in biocatalytic processes. *Chem. Eur. J.* **25**, 4871-4884, (2019).
71 Spadiut, O., Olsson, L. & Brumer, H., 3rd. A comparative summary of expression systems for the recombinant production of galactose oxidase. *Microb. Cell Fact.* **9**, 68, (2010).