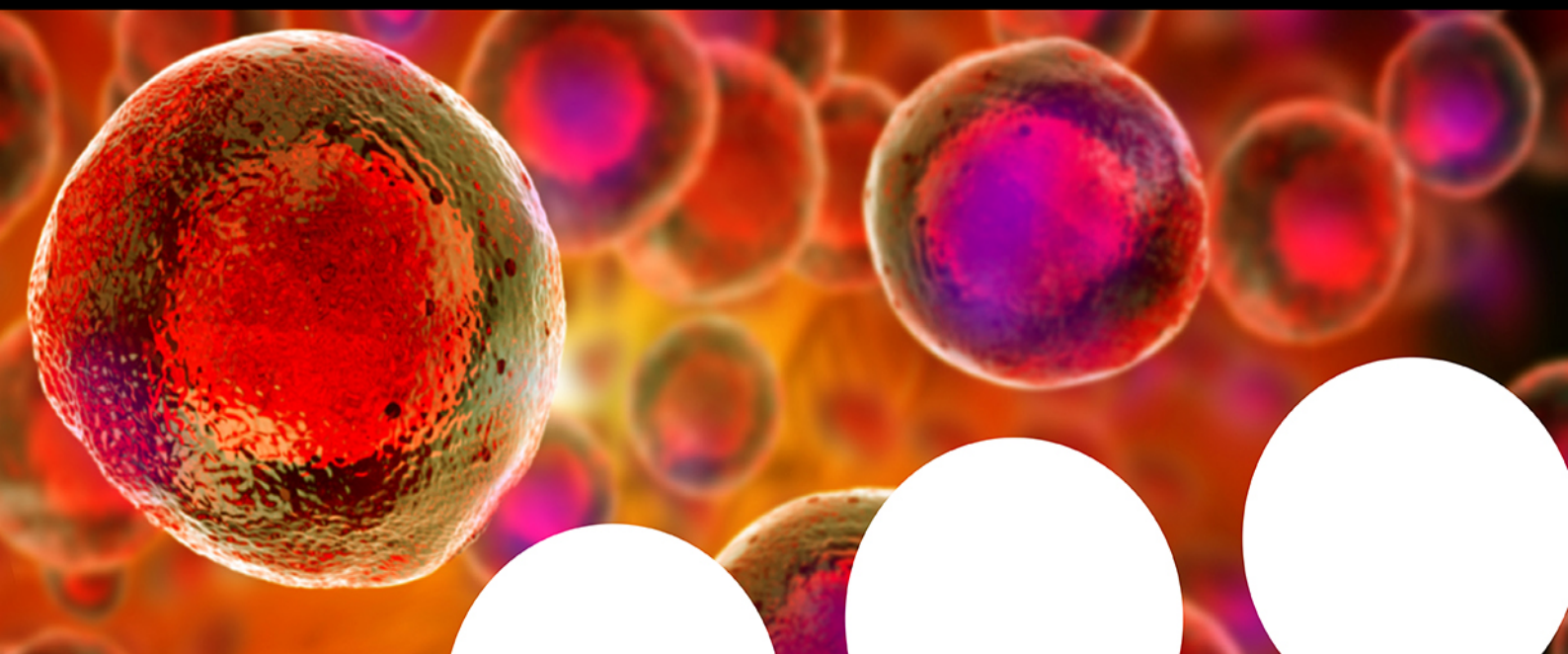


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Enzymatic Cascade for the Synthesis of 2,5-Furandicarboxylic Acid in Biphasic and Microaqueous Conditions: 'Media-Agnostic' Biocatalysts for Biorefineries

Milica Milić,^[a] Emil Byström,^[b] Pablo Domínguez de María,^{*[c]} and Selin Kara^{*[a, d]}

5-hydroxymethylfurfural (HMF) is produced upon dehydration of C6 sugars in biorefineries. As the product, it remains either in aqueous solutions, or is in situ extracted to an organic medium (biphasic system). For the subsequent oxidation of HMF to 2,5-furandicarboxylic acid (FDCA), 'media-agnostic' catalysts that can be efficiently used in different conditions, from aqueous to biphasic, and to organic (microaqueous) media, are of interest. Here, the concept of a one-pot biocatalytic cascade for production of FDCA from HMF was reported, using galactose oxidase (GalOx) for the formation of 2,5-diformylfuran (DFF),

followed by the lipase-mediated peracid oxidation of DFF to FDCA. GalOx maintained its catalytic activity upon exposure to a range of organic solvents with only 1% (v/v) of water. The oxidation of HMF to 2,5-diformylfuran (DFF) was successfully established in ethyl acetate-based biphasic or microaqueous systems. To validate the concept, the reaction was conducted at 5% (v/v) water, and integrated in a cascade where DFF was subsequently oxidized to FDCA in a reaction catalyzed by *Candida antarctica* lipase B.

Introduction

5-hydroxymethylfurfural (HMF) is considered to be one of the future key building blocks from biorefineries. HMF can be produced from C6 sugars originating from lignocellulose (mostly glucose), upon a triple acidic dehydration. In some cases, glucose is first isomerized to fructose, as the dehydration of fructose proceeds in a more straightforward manner. Glucose

from lignocellulose is typically found in water as a product of cellulose hydrolysis and thus, the produced HMF may remain in aqueous media or may be in situ extracted in an organic solvent (through a biphasic medium). Therefore, for the subsequent valorization of HMF, robust catalysts that show versatility and can perform reactions both in aqueous solutions and in (microaqueous) organic systems are highly desirable.

Biocatalysis is intuitively considered as a technology to be implemented in aqueous media, since the majority of biochemical processes occur in water. However, with the rise of biocatalysis in the 1980s, and the necessity to explore the versatility of the application of enzymes in different industrial processes, the initiative to use enzymes in non-conventional (non-aqueous) media arose, and proved to be successful.^[1,2] Advantages of the use of water-free media in biocatalysis are the greater stability of enzymes, and the straightforward recovery of the catalyst.^[3] In particular, the advantages of using water as a reaction medium are easily outweighed in the case of industrial processes, where enzymes typically catalyze non-natural organic substrates which have a higher solubility in non-aqueous media.^[4] Although a minimum amount of water is necessary for the hydration of the enzyme molecule, this is relatively low and no bulk quantities of water are needed.^[5,6] Non-conventional media encompass a wide array of potential reaction systems, ranging from ionic liquids (ILs), deep eutectic solvents (DES), neat solvents, microaqueous reaction systems (MARS), and biphasic systems consisting of water and organic media.^[7] Recent developments showed great potential of using enzymes in low-water media, which became not limited to lipases.^[8] When it comes to biorefineries, the possibility that enzymes can efficiently catalyze reactions in media containing different water proportions is clearly an asset, as it provides adaptation to varied (crude) effluent types.


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Galactose oxidase (GalOx, EC 1.1.3.9) gained momentum in recent years, mostly due to extensive research in the field of enzyme engineering.^[9] GalOx catalyzes the oxidation of primary alcohols to their corresponding aldehydes, using molecular oxygen as a cosubstrate.^[9] As in all other enzymes that need gaseous (co)substrates, the cosubstrate solubility in water (e.g., the solubility of O₂ is 0.25 mM at 20 °C) renders to reach high product titers. However, the solubility of gaseous substrates is higher in organic solvents than water, which opens up new process windows for the use enzymes in organic media.^[10] Despite the potential, only a handful of examples showcasing GalOx in non-conventional media have been reported so far.^[11,12] GalOx immobilized on an epoxy resin showed exceptional tolerance to several neat organic solvents, when used for oxidation of 3-fluorobenzyl alcohol.^[11] Additionally, engineered variants of GalOx have been used in their free form in the presence of various different organic cosolvents in aqueous media.^[12]

The substrate scope of GalOx is broad, and includes sugar-derived compounds, primary and benzyl alcohols, as well as HMF. Namely, HMF poses a great platform for the synthesis of different biobased and biomass-derived chemicals, most prominently 2,5-furandicarboxylic acid (FDCA). FDCA has been named one of the top twelve value-added chemicals from biomass.^[13] FDCA serves as a backbone of polyethylene furanoate (PEF), a material that poses a 100% biobased alternative to conventional polyethylene terephthalate (PET) plastic.^[14]

Since HMF contains an alcohol group and an aldehyde group, the biosynthesis of FDCA from HMF requires three consecutive oxidation steps, which means the reaction can take place through a myriad of intermediate products.^[15] A variety of processes for the oxidation of HMF to FDCA have been explored, including the use of electrocatalysis, photocatalysis, or catalysis using metal oxides.^[16]

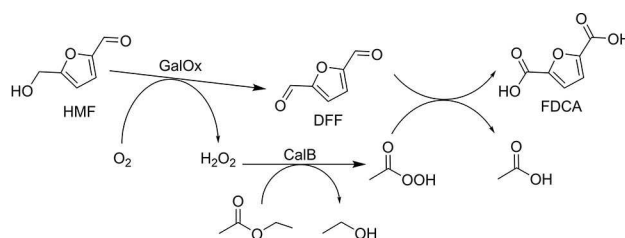
However, biocatalysis remains the golden standard of sustainable green production. One of the first cascades, albeit not completely biocatalytic, was proposed in 2013, in which HMF was first oxidized by 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to obtain DFF, which was then oxidized to FDCA using peracetic acid formed in situ with *Candida antarctica* lipase B (CalB).^[17] A combination of magnetic lipase and TEMPO as the mediator was successfully used to oxidize HMF to FDCA via 5-formyl-2-furoic acid (FFA) at ambient temperature and pressure.^[18] Most recently, the aforementioned chemoenzymatic approach was also combined with whole-cell biocatalysis using *Trichoderma reesei* filamentous fungi in order to obtain FDCA using 5-hydroxymethyl-2-furan carboxylic acid (HMFCFA) as the intermediate product.^[19] In another study, whole-cell biocatalysis was employed in a cascade reaction to transform HMF to FDCA using vanillin dehydrogenase (VDH1) and HMF/furfural oxidoreductase (HmfH) co-expressed in *Escherichia coli*.^[20] The possibility of using a single enzyme to catalyze the oxidation of HMF to FDCA was explored using the relatively recently discovered 5-hydroxymethylfurfural oxidase (HMFO).^[21] The same enzyme was used to convert HMF to FFA, which was then transformed into FDCA using CalB.^[22] *Pseudomonas putida* strain

S12 expressing HMFO was used for efficient whole-cell bio-transformation of HMF to FDCA.^[23] A tandem reaction combining *Escherichia coli* and wild-type *Pseudomonas putida* KT2440 resulted in a full conversion of HMF with high DFF yields.^[24] In a different approach, as HMF poses an inhibitor in the production of biofuels from lignocellulose waste, immobilized cells of *Burkholderia cepacia* H-2 were used to remove HMF from the process by transforming it to FDCA.^[25] Another proposed strategy was to use an aryl-alcohol oxidase (AAO) to transform HMF to FFA, subsequently transforming it to FDCA using an unspecific peroxygenase.^[26] Likewise, a one-pot cascade reaction using GalOx, horseradish peroxidase (HRP) and periplasmic aldehyde oxidase (PaoABC), with the same intermediate product was also established.^[27] Overall, the biocatalysis community has been witnessing great progress in the conversion of HMF to FDCA in different reaction systems via enzymatic or chemoenzymatic methods using isolated enzymes or whole-cells.

Although numerous biocatalytic synthesis routes to FDCA have been proposed, for their scalability further optimizations related to productivity and cost-effectiveness are typically needed.^[16] The tandem reaction utilizing GalOx and CalB for FDCA formation has been reported in the past in aqueous solutions. While being promising, the use of pure water-based media restricts its use to only aqueous effluents from biorefineries, and furthermore, it obliges to the separation of intermediate products, due to the incompatibility between the two reaction steps (oxidase catalysis and lipase-mediated peracid oxidations).^[28]

Given the needs of providing highly integrated systems for biorefinery, in which crude media – containing more or less water and impurities – can be used, herein, we propose a three-step bio-bio-chemocatalytic reaction cascade for the synthesis of FDCA (Scheme 1). The first step of the reaction is based on the oxidation of 5-hydroxymethylfurfural (HMF) to 2,5-diformylfuran (DFF) using GalOx. The enzyme uses molecular oxygen (O₂) as the cosubstrate, yielding hydrogen peroxide (H₂O₂) as the by-product. The H₂O₂ will be taken up by the second enzyme, CalB, which will utilize it to convert ethyl acetate into peracetic acid.^[17] The in situ formed peracetic acid will then oxidize DFF to FDCA.

In the case of chemoenzymatic cascades, the use of a biphasic or microaqueous system brings the best of both worlds: (i) water for the preference of enzymes, as well as (ii) an



Scheme 1. Conceptual enzymatic cascade for the synthesis of 2,5-furandicarboxylic acid (FDCA) from 5-hydroxymethylfurfural (HMF) through 2,5-diformylfuran (DFF) using galactose oxidase (GalOx) and *Candida antarctica* lipase B (CalB).

organic solvent for the preference of the hydrophobic substrates.^[29–31]

Results and Discussion

The primary focus of this study was to explore the 'medium engineering' scope for GalOx catalysis, with the aim of facilitating a smooth transition between the two steps of the reaction cascade, as well as potentially opening the possibility of establishing a one-pot cascade. The solvents used were chosen due to their different chemical structures, as well as physicochemical characteristics such as boiling point, water solubility, and hydrophobicity (log *P*) (Table S1).^[32] Cyclopentyl methyl ether (CPME) was chosen as the environmentally friendly and green(er) alternative to traditional solvents, which greatly contributes to the broader picture of establishing the production of biobased plastics.^[33,34] The motivation behind using ethyl acetate originated from the goal of using it as both a substrate and solvent component in potential following steps of the cascade.^[17] Additionally, lipases, catalyzing the second reaction step, exhibit the phenomenon of interfacial activation, meaning that they show higher activity in biphasic systems in comparison to pure aqueous conditions.^[35]

Effect of biphasic systems on galactose oxidase activity

Medium engineering can eliminate the need for extensive downstream processing for the isolation of intermediates. For this purpose, it was first necessary to assess the potential effects of organic solvents on the catalytic performance of GalOx. As pure water-based media have proven successful (although with downstream units), biphasic systems with 50% (v/v) of phosphate buffer were set as a starting point, and the range was consequently expanded with biphasic systems of lower water contents down to 1% (v/v), falling into the category of microaqueous systems (without bulk water quantities).^[19] The setup was analyzed with the preselected water-immiscible organic solvents of different physicochemical characteristics, with three of the most representative shown in Figure 1.

The presence of organic solvents in the system did not display a significant effect on the residual activity of GalOx over the course of 72 h. CPME, dodecane, and EtOAc are solvents with vastly different chemical structures, water miscibility and log *P* value. However, regardless of the values of the aforementioned parameters, the residual activities remained above the 80% mark in most cases. No visible correlation was found between the obtained results and the properties of the organic solvents, considering the physicochemical properties and their chemical structures. Moreover, the results corroborate that the log *P* value cannot be used as a sole criterion to predict biocompatibility of the solvent, given the complexity of establishing enzymatic reactions in biphasic media.^[36] The ten-fold reduction of the aqueous content in the biphasic system from 50% (v/v) to 5% (v/v) did not result in further loss of activity, as was the case for all investigated solvents.

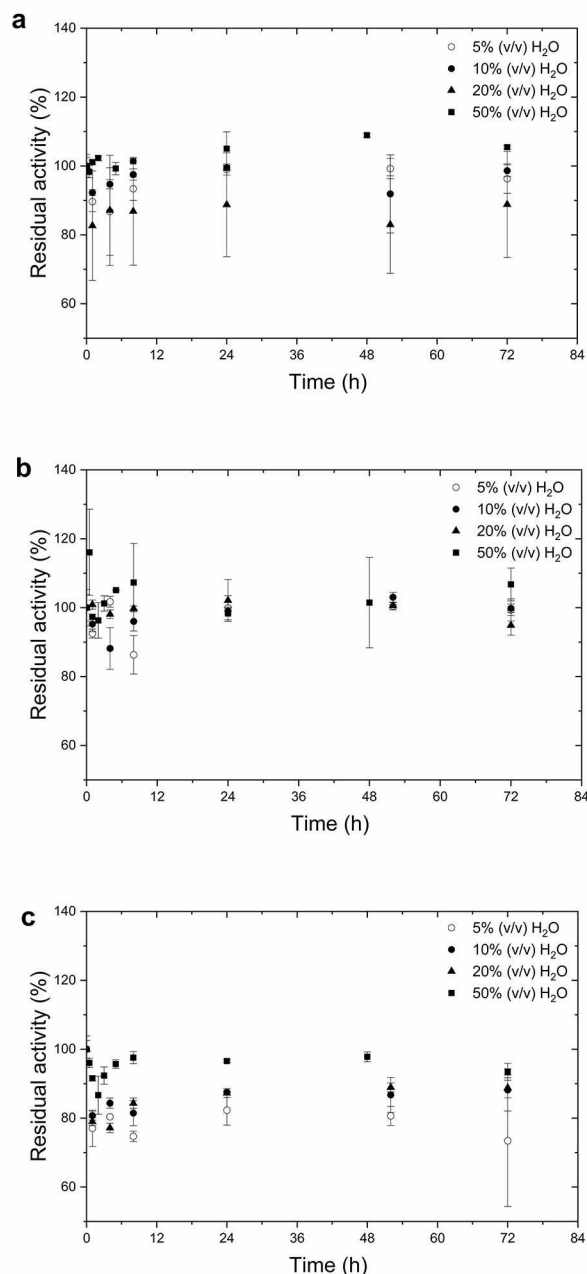


Figure 1. Residual activities of GalOx in biphasic systems with 50, 20, 10 and 5% (v/v) of aqueous phase and cyclopentyl methyl ether (CPME, a), dodecane (b), and ethyl acetate (EtOAc, c) at 1200 rpm and 25 °C measured over the course of 72 h using 100 mM D-galactose as the substrate. The residual activity is expressed relative to the initial activity of GalOx in 100 mM NaPi buffer at pH 7.4 and time zero. The experiments were performed in biological triplicates.

Effect of microaqueous systems on galactose oxidase activity

Encouraged by the obtained positive results even at water content as low as 5% (v/v), the subsequent reduction of the aqueous phase led the study into the area of microaqueous systems with 1% (v/v). The residual activity was monitored for 48 h, and the obtained results were highly comparable to those

obtained at the same timepoint with the other biphasic systems. The enzyme kept the highest activity in toluene, and the lowest in CPME, although residual activities higher than 70% were recorded in all cases (Figure 2).

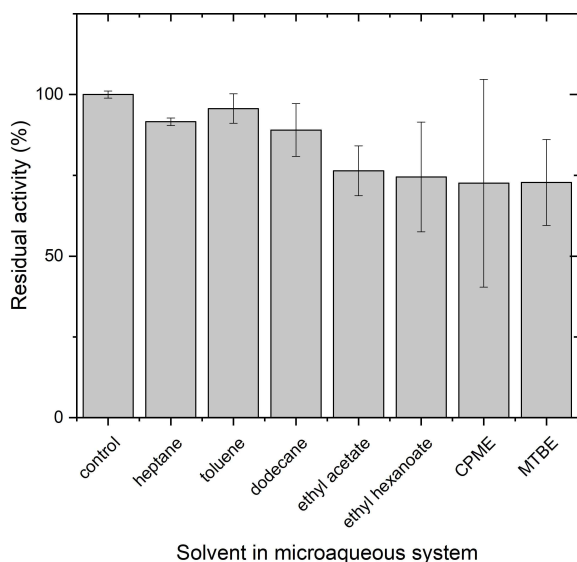


Figure 2. Residual activity of GalOx in microaqueous (two-liquid-phase) systems with various organic solvents and 1% (v/v) of aqueous phase, at 1200 rpm and 25 °C, measured after 48 h using 100 mM D-galactose as the substrate.

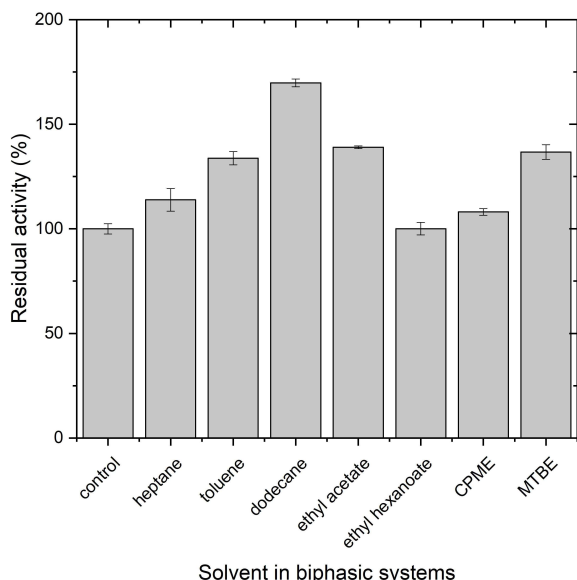


Figure 3. Residual activity of GalOx upon exposure to neat organic solvents for 20 h (monophasic system), and 2 h after subsequent reconstitution to biphasic systems with 50% (v/v) aqueous phase at 1200 rpm and 25 °C using 100 mM D-galactose as the substrate. The control experiment represents the enzyme without exposure to organic solvents.

Effect of neat solvents on galactose oxidase activity

Inspired by the results, the GalOx stability was also evaluated in neat as well as in water-saturated solvents, but no catalytic activity could be observed. It must be noted, however, that the exposure to solvents does not affect the activity of the enzyme, as shown in previous works where both free- and immobilized GalOx exhibited significant tolerance towards organic solvents.^[11,12] To corroborate this, upon 20 h of exposure to the aforementioned solvents, buffer was added to the reaction vessels. Thus, the systems were reconstituted to biphasic systems with 50% (v/v) aqueous phase, and the activity was measured after two hours of equilibration (Figure 3).

In some cases, the exposure to neat solvents and regeneration of GalOx in 50% (v/v) H₂O biphasic system led to a remarkable increase in activity. A similar observation was made for decarboxylation of ferulic acid, where the enzyme exhibited higher stability (3.5-fold increase in half-life time) in biphasic systems compared to aqueous media.^[37] Importantly, the results confirm that the exposure to organic solvents does not have a detrimental effect on the enzyme, and that the enzyme retains sufficient activity to be used for subsequent catalysis.

Oxidation of HMF to DFF in EtOAc-based biphasic and microaqueous media

As previously underlined, the actual motivation for exploring the stability of GalOx in the presence of organic media is to use the enzyme in a multistep chemo-enzymatic reaction for the synthesis of FDCA (Scheme 1). Therefore, the oxidation of 1 mM HMF to DFF was systematically studied in EtOAc based biphasic systems of various water contents (at 1 mL scale), using GalOx, with addition of auxiliary enzymes: horseradish peroxidase and/or catalase (Table S2). HRP was added as an activator, whereas catalase was added to remove H₂O₂. The highest conversion of HMF was 58.6 ± 3.6%, achieved when all three enzymes were used in pure buffer. The conversions in biphasic and microaqueous systems with ethyl acetate were lower, however, the reduction in the aqueous phase content from 50% (v/v) to 1% (v/v) did not result in a significant reduction in conversion, providing operational options for the water-free biocatalytic cascade to FDCA.

Catalysis using GalOx requires a careful interplay of different process parameters. The active site of GalOx contains a tyrosine radical bound to a copper(II) ion, which is reduced to a nonradical tyrosine copper(I) complex during oxidation of the substrate.^[38] This tyrosine radical can also undergo a reduction to a nonradical copper(II) complex, which is inactive.^[39] Therefore, the enzyme requires a single-electron oxidation in order to regenerate the active site.^[39] Although extensive studies have been made to find alternative activator molecules, such as potassium ferricyanide (K₃[Fe(CN)₆]), the most commonly used activator remains horseradish peroxidase (HRP).^[40] Based on the results shown in Table 1, the addition of HRP indeed had a positive effect on the reaction yield, especially noticed in the

Table 1. Conversion of HMF to DFF in EtOAc-based biphasic systems with 50, 20, 10, 5, and 1% (v/v) of 0.1 M NaPi buffer at pH 7.4, as well as pure buffer using GalOx and HRP and/or catalase as auxiliary enzymes.

Added aqueous phase [% (v/v)]	Conv. ^[a] [%]	Conv. ^[b] [%]	Conv. ^[c] [%]	Conv. ^[d] [%]
1	2.6 ± 0.1	4.6 ± 0.4	3.7 ± 1.0	1.9 ± 0.2
5	6.7 ± 0.4	11.5 ± 1.7	8.0 ± 0.7	6.9 ± 1.6
10	7.6 ± 0.6	11.5 ± 0.7	7.6 ± 0.2	5.6 ± 1.8
20	8.8 ± 1.5	15.6 ± 0.4	10.0 ± 0.5	6.6 ± 1.0
50	15.0 ± 0.8	26.1 ± 0.2	16.5 ± 1.6	9.2 ± 0.4
100	24.9 ± 1.4	36.7 ± 0.6	33.0 ± 1.8	25.6 ± 1.3

[a] 2 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, 1 mg mL⁻¹ catalase. [b] 4 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, 1 mg mL⁻¹ catalase. [c] 4 mg mL⁻¹ GalOx, 2 mg mL⁻¹ HRP, 1 mg mL⁻¹ catalase. [d] 4 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, 2 mg mL⁻¹ catalase. All reactions were performed at 25 °C, 200 rpm for 72 h and subsequently analyzed using HPLC. All reactions were performed in biological triplicates.

reaction performed in pure buffer, both when added to GalOx alone, or in presence of catalase as well.

Hydrogen peroxide (H₂O₂) is the by-product of GalOx-catalyzed oxidation, however, it has been shown to both inhibit and deactivate the enzyme.^[41] Namely, hydrogen peroxide is not soluble in organic media, which means that its full amount is confined to the aqueous phase. Hence, the concentration of H₂O₂ increases with the decrease in water content in the biphasic system. In order to alleviate this, catalase was added to the reaction to dismutate H₂O₂ into H₂O and O₂.^[38] The addition of catalase alone resulted in much lower DFF yields, in the case of pure buffer as a reaction medium. Interestingly, in the case of biphasic systems the obtained yields with the addition of catalase alone, were lower than those achieved in catalase-free systems, or where it was present along with HRP.

Oxygen serves as a cosubstrate for GalOx, and it is therefore necessary to facilitate enough O₂ in the system. Assuming the saturation of the enzyme with the substrate, as well as atmospheric pressure conditions, the reaction rate will be almost directly proportional to the oxygen concentration.^[38] Although some oxygen is provided, or more precisely, can be recycled from the breakdown of H₂O₂ by catalase, it is necessary to provide additional oxygen for the process. In higher scale reactors this can easily be achieved by bubbling.^[38,42] In low volume reactions, one possible solution is to maximize the headspace volume to reaction volume ratio within the reaction vessel.^[32]

Taking all these aspects into account, a combination of galactose oxidase, horseradish peroxidase, and catalase resulted in the highest HMF conversions when comparing the results obtained in pure buffer (Table 1). Therefore, based on the screening experiments, further optimization of the reaction ensued. In experiments that followed, all three enzymes were used in all trials, however, at different concentrations, in order to assess the optimal ratio of the enzymes.

A comparison of the results obtained with 2 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, and 1 mg mL⁻¹ catalase revealed that the increase in headspace, and therefore, oxygen availability in the system, resulted in a higher observed conversion of HMF to DFF in the case of all biphasic systems. An interesting result can be

seen in the case of the reaction performed in 100% buffer, where the introduction of the headspace volume resulted in a nearly halved yield to the previously recorded result. When comparing the results obtained with different enzyme combinations, the achieved results are significantly better in all four cases, in comparison with the initial results. The combination of 4 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, and 1 mg mL⁻¹ catalase fared best, resulting in the highest DFF yields in all biphasic systems, as well as pure buffer.

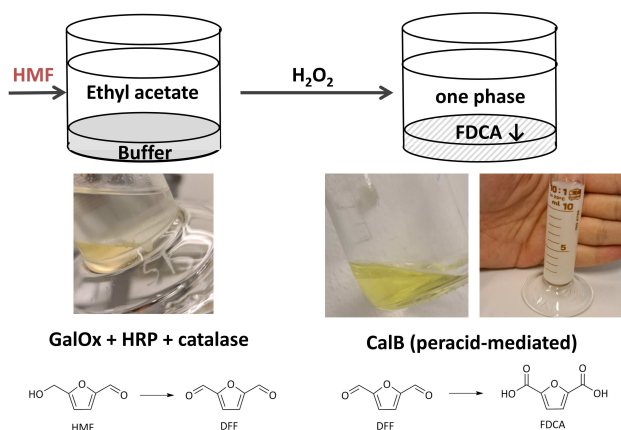
Scale-up towards FDCA synthesis

Once the operational conditions were set, the subsequent step was performing the reaction at preparative conditions. For this, the reaction was performed using the enzyme concentrations that showed the most promising results (the combination of 4 mg mL⁻¹ GalOx, 1 mg mL⁻¹ HRP, and 1 mg mL⁻¹ catalase). With the goal of minimizing the water content, but at the same time maintaining sufficient productivity of the reaction, the biphasic system of choice was ethyl acetate containing 5% (v/v) of buffer. The total reaction volume was 0.1 L and it was performed in 2 L glass bottles in order to keep roughly the same liquid-to-headspace ratio at 150 rpm.

Close examination of the scaled-up reaction revealed that, contrary to extensive literature search, the oxidation of HMF to DFF is much faster than anticipated (~20% yield in 3 h), with the majority of DFF produced in the initial few hours (Figure S2). A decrease in the residual activity was observed with the increase of the concentration of DFF in a biphasic system (Figure S3). Dialdehydes, such as DFF, can interact with the surface amino acids of the enzyme. The most prominent example is glutaraldehyde, a crosslinking molecule commonly used for enzyme immobilization.^[43,44] The DFF partitioning to the aqueous phase was also noticed, and it was especially visible at higher concentrations. At 30 °C its saturation concentration (maximum solubility) in water is approximately 15 g L⁻¹ (~120 mM), whereas at temperatures below 20 °C DFF tends to precipitate, and therefore can easily be separated from HMF and removed from the aqueous solution.^[45]

Upon 72 h, CalB was added directly into the reaction, along with H₂O₂ (stepwise) and the reaction temperature was elevated to 40 °C. Remarkably, upon 24 h of reaction, the two phases disappeared, and a uniform liquid phase was formed, together with a white precipitate, which was confirmed to be FDCA by HPLC analysis. FDCA has a relatively low solubility in water and ethyl acetate in comparison to other solvents.^[46] Moreover, the acidic conditions (pH 3) facilitated by the presence of (per)acetic acid caused FDCA to precipitate as also documented in the literature.^[47]

Thus, the herein established bio-bio-chemocatalytic concept may provide useful operational options for straightforward downstream processing when using the biphasic system or the microaqueous approach (Scheme 2).



Scheme 2. Conceptual approach for the biphasic (or microaqueous) system for the synthesis of FDCA from HMF.

Conclusion

Galactose oxidase showed an excellent stability in a myriad of organic solvents, retaining its activity upon exposure to the solvents in biphasic systems or in media with reducing water contents (microaqueous). Therefore, the oxidation of HMF to DFF could be successfully established in ethyl acetate-based biphasic/microaqueous systems with water contents as low as 1% (v/v). The reaction was successfully scaled up to a 100 mL scale, with only a minor decrease in the conversion of HMF to DFF. Following this, the reaction was coupled to a second step, namely the CalB-catalyzed synthesis of peracetic acid, which in situ oxidizes DFF to FDCA. Due to the low solubility of FDCA both in ethyl acetate and water, the products precipitated, simplifying the downstream processing.

The herein presented approach showed relevance for biorefineries, because the use of 'media-agnostic' biocatalysts enables the synthesis of FDCA in a broad range of conditions, from pure aqueous media to other systems without bulk water added. Given the many types of (crude) effluents expected in biorefineries, having these catalysts in hand may be of high importance, as they can be straightforwardly adapted to on-demand conditions.

Once the proof-of-concept has been shown, further optimization steps need to be considered to reduce reaction times and improve overall yields and productivities. Apart from fine-tuning the different enzyme proportions and loadings, it must be noted that the concept has been demonstrated with a commercial wild type enzyme preparation of GalOx, and not with other variants (e.g., the M₃₋₅ mutant), which has been reported to display a higher affinity towards HMF as the substrate.^[12] Likewise, from the standpoint of reaction engineering, improving the mixing conditions, in particular, providing the optimal amount of oxygen, are aspects to be considered. When it comes to the overall cascade, the tendency of FDCA to precipitate due to its insolubility in the reaction medium opens new frontiers towards easier downstream processing. Overall, the versatility of the reaction media, together with straightfor-

ward FDCA recovery may be important assets for the implementation of the synthetic route in future biorefineries.

Experimental Section

Chemicals

Horseradish peroxidase was purchased from PanReac AppliChem ITW Reagents (Germany). DFF and FDCA were purchased from TCI Chemicals (Japan). ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was purchased from Roche (Germany). D-Galactose ($\geq 98\%$) was purchased from Alfa Aesar (United States). Hydrogen peroxide was purchased from Roth (Germany). A liquid preparation of CalB was generously received from c-LEcta (Germany). All other chemicals were purchased from Sigma-Aldrich (Germany) or VWR (Germany) and used as received.

Activity assay

For measuring the activity of galactose oxidase, a modified ABTS-HRP coupled assay was used. In this reaction, D-galactose or HMF is oxidized to D-galacto-hexodialdose or DFF, respectively, using galactose oxidase, yielding hydrogen peroxide (H₂O₂) as the byproduct. The H₂O₂ is taken up by horseradish peroxidase which utilizes it to oxidize ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to its respective cation radical. A solution containing 1 mM ABTS, 100 mM D-galactose, and 2 U mL⁻¹ of horseradish peroxidase was prepared in 100 mM NaPi buffer at pH 7.4. 10 μ L of the enzyme solution were added to 990 μ L of the assay mixture, and the change of absorbance over time was measured at 405 nm and 25 °C for 1 min. The molar extinction coefficient of ABTS was previously determined to be 30.9 L \times min \times mmol⁻¹. One Unit of GalOx activity was defined as the amount of enzyme that is necessary for the oxidation of 2 μ mol of ABTS per minute, which equals the consumption of 1 μ mol of O₂ per minute, under the conditions described above.^[48] The residual activity is expressed relative to the initial activity of galactose oxidase in 100 mM NaPi buffer pH 7.4 at time zero. The measurements were performed in biological triplicates.

Optimization of the oxidation of HMF to DFF

In the first optimization step, the initial concentration of HMF was increased from 1 mM to 50 mM. Along with increasing the HMF concentration, ensuring a higher oxygen availability was of utmost importance. Therefore, the reaction was performed in 35 mL vials, with the headspace volume of 33.5 mL. The glass reaction vials were also opened every two hours for the first eight hours of reaction in order to introduce fresh oxygen into the system. A consequence of changing the reaction vessels led to the reduction of shaking speed to 200 rpm, as the reaction was now performed in an incubator.

Scale-up and coupling

As there was no significant difference between the results obtained in a 1% (v/v) and 5% (v/v), the scale-up and coupling to the second reaction step were performed in ethyl acetate with 5% (v/v) aqueous phase with 4 mg mL⁻¹ galactose oxidase, 1 mg mL⁻¹ HRP and, 1 mg mL⁻¹ catalase, the combination of enzyme concentrations that fared best. The 0.1 L reaction was performed in 2 L laboratory flasks at 25 °C and 150 rpm in the incubator. Upon 72 h, 75 μ L of liquid CalB of 1.15 g mL⁻¹ was added directly to the biphasic

system, and the temperature was increased to 40 °C. In addition, stepwise addition of hydrogen peroxide ensued, with 250 µL of a 30% aqueous solution of H₂O₂ was added hourly for the first six hours of reaction, starting at time point zero. Upon the addition of hydrogen peroxide foam formation was observed, presumably due to the activity of catalase. As a consequence, the reaction vessel filled with oxygen during the first hour of reaction. After three hours of reaction, a vinegar-like odour could be noticed, indicating the presence of peracetic or acetic acid.

HPLC sample preparation

The HPLC samples for measuring the yield of DFF were prepared by taking a 10 µL sample from both the aqueous and organic phase and quenching the reaction with an equal amount of 0.5 M H₂SO₄. The sample was then diluted with 480 µL of buffer or ethyl acetate and analyzed. In the case of FDCA, a 200 µL sample of the reaction mixture was quenched by adding the equal amount of 0.5 M H₂SO₄ and analyzed without dilution. As FDCA seems to precipitate, the reaction vessel was purged with 12 mL of methanol, and the sample was prepared in the same manner.

HPLC analysis

HPLC analysis was done using an Agilent Technologies (Germany) 1260 Infinity II high-performance liquid chromatography system consisting of a G7111B quaternary pump, a G7129A vial sampler, a G7116A column thermostat, and a G7117C diode-array detector (DAD) detector coupled with a G6125C LC-ESI-MS detector. Separation of analytes was performed on a Rezex™ column (ROA, organic acid H+ 8%, 300×7.8 mm) from Phenomenex (Germany).

The mobile phase, 0.05 N H₂SO₄ in Milli-Q water was run at a flow rate of 0.75 mL min⁻¹ at 50 °C and isocratic conditions.^[49] The injection volume was 10 µL and the HPLC run was 40 min. The detection wavelength used was 280.4 nm for HMF and DFF, and 270.4 nm for FDCA. The standards used were HMF, DFF and FDCA with their respective chromatograms shown in Figure S4.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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