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Selective Oxidation of 5-Hydroxymethylfurfural to 2,5-Diformylfuran in Biphasic Media using Immobilized Galactose Oxidase: Proof of Concept and Limitations

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The oxidation of 5-hydroxymethylfurfural (HMF) to 2,5-diformylfuran (DFF) is a key reaction in valorizing biomass. DFF is hardly soluble in water, while HMF is often obtained from biorefineries in crude wet organic fractions. Thus, the reaction is challenging for both biocatalysis performed in aqueous media, and for chemocatalysis where the presence of water often results in catalyst poisoning. Galactose oxidase (GalOx) can selectively oxidize HMF to DFF and displays promising activity in aqueous-organic media. In this study, GalOx was immobilized on ten carriers, assessing the immobilization yield, activity, and stability. Covalently immobilized GalOx catalyzed the oxidation of HMF to DFF in neat and water-saturated EtOAc, and in biphasic systems of various water contents. At 50% v/v H_2O, the reaction was conducted at a semi-preparative scale (50 mL) with no adverse effect on DFF yield. Some limitations arise, such as enzyme deactivation, and adsorption of DFF to the support, particularly in the aqueous fraction. Future options to upgrade the route may include designed stable enzymes under the presence of HMF/DFF, and the setup of microaqueous systems where DFF adsorption is minimized. The use of wet EtOAc media would be a promising approach in future biorefineries employing inexpensive crude wet organic fractions.

Introduction

Furan-based chemicals represent a promising platform for biomass valorization, given the numerous possibilities for their functionalization and synthesis. Among these, 5-hydroxymethylfurfural (HMF) and its derivatives have shown various applications – ranging from pharmaceuticals and flavors, to solvents, biofuels, and polymer building blocks.[1–3] 2,5-diformylfuran (DFF), obtained through HMF oxidation, is used in the pharmaceutical industry, as a precursor of resins, or to synthetize polymers and other secondary furanic compounds, most notably 2,5-furandicarboxylic acid (FDCA). [4,5] However, both HMF and DFF are inherently highly reactive, making the selective route to DFF rather challenging. Moreover, in biorefineries, HMF is commonly extracted from a biphasic system into a (water-saturated) crude organic fraction.[6–8] This may be particularly challenging for chemocatalysts, which typically need inert, non-aqueous conditions to properly perform reactions. To save costs, however, the use of crude effluents in biorefineries is prioritized, as simplified downstream units would be needed. Herein, the incorporation of robust "media-agnostic" biocatalysts, displaying activity in biorefinery-like crude media would be advantageous.[9] Moreover, immobilizing enzymes would render biocatalysts with higher stability under severe conditions,[10] while facilitating their recovery and reuse, and further simplifying downstream processing and improving cost-effectiveness.[11,12]

The oxidation of HMF to DFF is catalyzed by galactose oxidase (GalOx), a mononuclear copper oxidoreductase that uses molecular oxygen to oxidize primary alcohols to their respective aldehydes. GalOx has been widely used in electrochemical biosensors for the detection of galactose, used in the diagnosis of galactosemia and galactose intolerance [13][14]. For this purpose, GalOx has been immobilized on cellulose acetate membranes,[15] natural materials such as eggshell,[16] collagen,[17] and laponite clay,[18] ZnO nanorods,[19] or entrapped in a poly (glycidyl methacrylate-co-vinylferrocene) (poly (GMA-co-VFc)) film.[20] More recently, GalOx has been immobilized for its use in synthetic biotransformations, e.g. in alginate beads to synthesize 1,2,3-triazoles via click chemistry.[21] To achieve increased storage stability, GalOx was immobilized on hydrazide-modified cellulose microporous beads and microspheres made from hydroxyethyl methacrylate and ethylene dimethacrylate

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(poly(HEMA-co-EDMA).[22] In another approach, GalOx was immobilized onto silica beads via adsorption and covalent attachment.[23] GalOx immobilized on mesoporous silica particles displayed increased thermal resistance to 60 °C.[24] Immobilization of GalOx on chemically-modified porous silica beads enabled its use in a packed bed reactor.[25] Concerning biorefinery-oriented approaches, GalOx was encapsulated using Cu (II) and Zr (IV) to form nanoflowers, which were then used in the oxidation of HMF.[26] Nanoflower matrices based on $Cu_3(PO_4)_2$ were also used to co-immobilize galactose oxidase, horseradish peroxidase, and catalase, to obtain a more active and inhibition-resistant biocatalyst for oxidation of HMF to DFF.[27] More recent examples use immobilized GalOx as one of the enzymatic steps for the synthesis of islatravir (an HIV treatment drug).[28] GalOx was immobilized on commercial agarose carriers and acrylamide resins, using Ni-NTA affinity binding,[29] and also on agarose and methacrylate carriers to be subsequently integrated in a flow reactor setup.[30] In another recent study, GalOx was immobilized on Purolite® Lifetech™ ECR8285, leading to a biocatalyst with improved storage and thermal stability for the oxidation of 3-fluorobenzyl alcohol to be performed in various organic solvents.[31]

Despite these works, however, the use of immobilized GalOx in crude, water-saturated biorefinery-relevant conditions have received little attention hitherto. Developing heterogeneous GalOx that can perform the oxidation of HMF to DFF in real non-purified effluents may open an opportunity to valorize HMF under highly selective conditions, avoiding by-product formation. This paper explores options for immobilized GalOx in different media – from water-saturated ethyl acetate to biphasic systems – to validate the idea, and to pinpoint potential hurdles for its future implementation.

Results and Discussion

Carrier screening for immobilization

Covalent immobilization of a commercial preparation of galactose oxidase (GalOx) was investigated by comparing ten different methacrylate-based carriers. The Lifetech™ carriers by Purolite® are available in two preparations as macroporous resins, functionalized either with amino or epoxy groups. Moreover, the amino-functionalized carriers are present in two different spacer lengths (C2 and C6), each available in three different levels of porosity. Before immobilization, the resin needs to be activated with glutaraldehyde, which serves as a crosslinker molecule between the amino groups on the surface of the carrier, and the unprotonated amino groups of the enzyme molecule,[32,33] The epoxy-functionalized carriers come in four variants of different porosity, with ECR8285 having both epoxy and butyl groups on the surface, which make up a carrier with additional stability towards organic solvents.[34] The epoxy carriers do not require an activation step, and allow for multipoint covalent attachment.[35] The surface amino groups on the protein can, in a nucleophilic attack, open the epoxydic ring and establish a covalent bond.

Immobilization of GalOx on amino carriers showed promising results in terms of immobilization yields (Table 1). The highest immobilization yields were reported for ECR8304F and ECR8404F, two amino-functionalized carriers with the smallest pore size (300-600 Å). Likewise, the yields of GalOx immobilized on carriers with amino groups on a short spacer molecule were low, with the highest activity yield amounting to 8.2 ± 1.4 % in the case of ECR8304F. Thus, in the case of GalOx, the activity yield decreases when supports with higher pore diameter are used, exhibiting an effect opposite to the immobilization of phenolic acid decarboxylase on amino carriers.[36] On the other hand, GalOx immobilized on amino carriers with longer spacer molecules displayed no activity, albeit relatively high immobilization yields were reached. A similar trend regarding spacer length was previously observed in the immobilization of unspecific peroxygenase onto the investigated carriers.[37] A plausible explanation, given the high immobilization yield, is that the enzyme is, in fact, immobilized on the carrier, but either through residues that play a role in the active site, or in an unfavorable configuration that occludes the active site resulting in diminished activity. Moreover, the long spacer molecule allows for higher conformational flexibility of the enzyme, rendering the protein more susceptible to unfolding under the immobilization conditions.

Conversely, the immobilization of GalOx on epoxy carriers yielded slightly lower immobilization yields than the ones reported for amino-functionalized carriers, but the overall activity yields were higher. ECR8215F (epoxy) showed a distinct advantage in terms of activity yield with 28.3 ± 0.6 %, while the other three carriers showed similar promising results. Opposite to the amino-functionalized carriers, in the case of epoxy-functionalized carriers the activity yield increases with the increase in pore diameter, for the three carriers of comparable bead sizes, consistent with previous studies conducted with unspecific peroxygenase and a glycosyltransferase from *Polygonum tinctorium*.[37,38]

The reported high activity yields (Table 1) are obviously of importance but cannot be the sole parameter for choosing the most suitable carrier. Other aspects, such as increased biocatalyst stability need to be assessed as well. Therefore, the storage stability of epoxy-functionalized carriers was assessed by tracking the activity loss during four weeks of storage at 4 °C (Figure 1). GalOx immobilized onto ECR8285 (butyl/epoxy) retained 65.2 ± 12.9 % of its initial activity after four weeks, followed by GalOx immobilized onto ECR8209F (epoxy) and ECR8215F (epoxy), with 47.3 ± 6.4 % and 47.5 ± 4.9 %, respectively. Although ECR8285 showed the best relative storage stability, ECR8215F exhibited the highest activity yield, as well as the highest absolute activity after four weeks of storage and was therefore used in subsequent experiments for the actual oxidation of HMF to DFF.

Table 1. Parameters quantifying the efficiency of immobilization of GalOx on amino- and epoxy-functionalized Lifetech™ carriers along with the physico-chemical properties of each carrier.

Carrier	Functional group	Spacer length	Particle size (μm)	Pore diameter (Å)	Protein loading ($mg_{enzyme}/g_{carrier}$)	Immobilization yield (%)[a]	Specific activity (U/mg_{enzyme})	Activity yield (%)[b]
ECR8204F	epoxy	–	150–300	300–600	3.9 ± 0.8	52.0 ± 8.3	0.8 ± 0.1	14.6 ± 0.7
ECR8209F	epoxy	–	150–300	600–1200	4.0 ± 1.2	61.6 ± 8.4	0.9 ± 0.1	17.7 ± 2.9
ECR8215F	epoxy	–	150–300	1200–1800	3.8 ± 0.5	60.5 ± 9.1	1.0 ± 0.1	28.3 ± 0.6
ECR8285	butyl/epoxy	–	250–1000	450–650	2.2 ± 0.1	70.0 ± 1.0	1.3 ± 0.1	19.3 ± 0.2
ECR8304F	amino	short (C2)	150–300	300–600	3.9 ± 1.5	81.9 ± 2.0	0.3 ± 0.2	8.2 ± 1.4
ECR8309F	amino	short (C2)	150–300	600–1200	4.6 ± 2.6	69.4 ± 21.4	0.3 ± 0.2	6.4 ± 1.7
ECR8315F	amino	short (C2)	150–300	1200–1800	1.9 ± 0.8	65.6 ± 4.4	0.4 ± 0.1	4.7 ± 0.7
ECR8404F	amino	long (C6)	150–300	300–600	4.1 ± 1.8	68.8 ± 22.1	–	–
ECR8409F	amino	long (C6)	150–300	600–1200	3.8 ± 1.8	57.3 ± 15.4	–	–
ECR8415F	amino	long (C6)	150–300	1200–1800	3.7 ± 1.9	58.0 ± 14.0	–	–

[a] $Immobilization\ yield\ (\%) = \frac{m_{offered\ enzyme}\ (g) - m_{total\ enzyme\ in\ supernatants}\ (g)}{m_{offered\ enzyme}\ (g)}$; [b] $Activity\ yield\ (\%) = \frac{Activity_{immobilized\ enzyme}\left(\frac{U}{g_{wet\ carrier}}\right) \times m_{wet\ carrier}\ (g)}{Activity_{free\ enzyme}\left(\frac{U}{mg_{enzyme}}\right) \times m_{offered\ enzyme}\ (g)}$

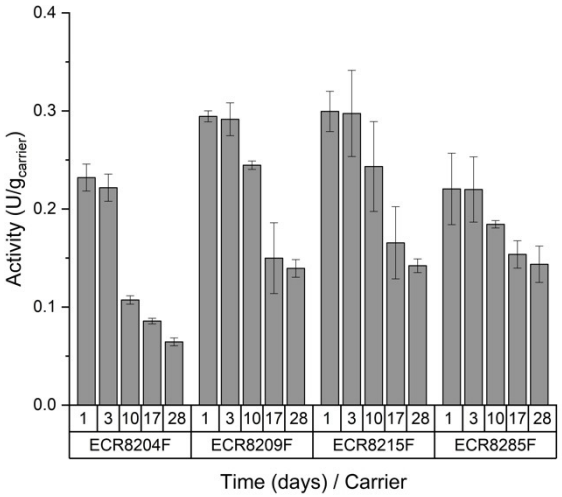

Figure 1. Storage stability of GalOx immobilized on different epoxy-functionalized Purolite® Lifetech™ carriers. The activity is expressed relative to the activity of the immobilized enzyme on day one. Reaction conditions: 1 mM ABTS, 100 mM D-galactose, 2 U mL^{-1} horseradish peroxidase in 0.1 M NaPi buffer pH 7.4, 10 mL reaction volume, 5 mg of immobilized enzyme at 25 °C. 1 mL samples were taken every 20 seconds (five samples in total) and single absorbance measurements were performed at 405 nm and 25 °C. The experiment was performed in biological duplicates.

Oxidation of HMF to DFF in different water-containing ethyl acetate-based media

A recent study showed that DFF can be used instead of glutaraldehyde as a crosslinking molecule in enzyme immobilization.[39] In that case, the immobilization of glucoamylase was performed on a polymethylmethacrylate support, where the resin was activated with DFF in buffer. To assess whether adsorption of HMF or DFF to the carrier could occur, these chemicals were incubated with ECR8215F (epoxy) in 0.1 M NaPi buffer pH 7.4 and ethyl acetate (EtOAc) (Figure 2). EtOAc would be an excellent reaction medium since almost no adsorption was detected for both HMF and DFF (Figure 2). In the case of buffer, however, around 15% of HMF was bound to the carrier after 72 hours. The highest level of adsorption was recorded for DFF in buffer, where almost 40% of the DFF in solution was lost to the carrier in the first 24 hours. After that, there was little to no additional adsorption, as the critical concentration was probably reached. Therefore, when using this type of carriers, the observed DFF yields may be lower than the actual ones, which needs to be considered when implementing the mass balance of the reaction. A biphasic system would enable a solution for both, since EtOAc would facilitate increased substrate and product solubility, whereas the immobilized enzyme would remain in the aqueous phase, shielded from the detrimental adsorptive effects of DFF.

As stated above, GalOx has previously displayed outstanding tolerance towards a variety of organic solvents. [9,40] Moreover, oxidation of HMF to DFF using GalOx in aqueous media, with various amounts of EtOAc as co-solvent has been shown. However, when the enzyme was used in its free form in neat EtOAc, no conversion of HMF to DFF was detected. This can be

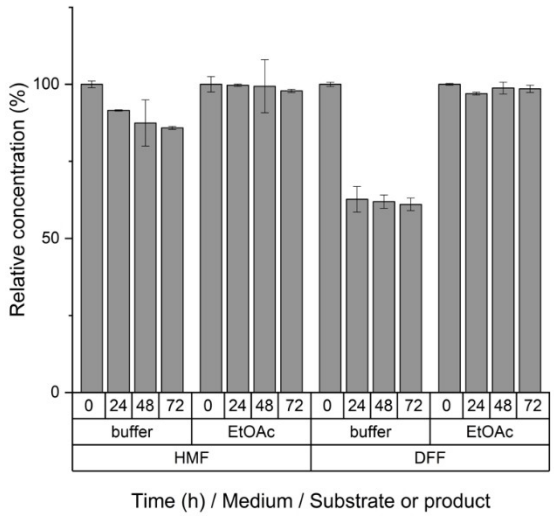

Figure 2. Adsorption of HMF and DFF onto Purolite® Lifetech™ ECR8215F epoxy-functionalized enzyme carrier in 0.1 M NaPi buffer at pH 7.4 and neat EtOAc. Reaction conditions: 0.250 g ECR8215F, 50 mM HMF or DFF, 3 mL total liquid volume in 30 mL reaction vials, 25 °C, 170 rpm, and 72 hours. All reactions were performed in biological triplicates and the residual amount of HMF/DFF in the liquid volume was subsequently analyzed using HPLC.

attributed to: (i) enzyme aggregation in organic media which creates a non-optimal diffusion-limited heterogenous system;[41] and (ii) high water-stripping capacity of EtOAc due to its high polarity (log $P < 2$).[42–44] Therefore, using immobilized GalOx would create a heterogenous system with less diffusion limitations, by supressing the agglomeration, while displaying higher process stability in the presence of water-saturated EtOAc mixtures. To validate the idea, the oxidation of HMF to DFF was performed using galactose oxidase immobilized on ECR8215F (epoxy), in an array of aqueous-organic media conditions (Figure S1). Using the immobilized enzyme, the reaction was successfully performed in both neat and water-saturated EtOAc, with no significant difference in DFF yield between the reactions performed in EtOAc with externally added 5% v/v H_2O and lower water contents. Regardless of the amount of added aqueous phase, in most cases, the reaction reached a plateau at 48 h (Figure S1). The highest overall yields were achieved in a 50:50 v/v ratio of EtOAc and buffer, reaching a yield of 2.9 ± 0.4 % after 72 hours of reaction, and 9.0 ± 0.8 % after another 72 hours upon exchanging the carrier for a fresh batch. However, an observed limitation at non-aqueous media (namely wet EtOAc) was that the immobilized enzyme tended to cluster around the scarce water molecules available, forming spherical agglomerates (Figure S2). This effect was most prominent in water-saturated and neat EtOAc, where the only additional water is introduced through the "wet" carrier. This results in occlusion of the enzyme molecules immobilized on the carrier particles that make up the centre of the sphere, making them unavailable to reach the substrate molecules, and therefore resulting in lower yields. Considering that a substantial amount of DFF (approximately 40% after 24 hours) is lost to adsorption onto the carrier, the actual amount of produced DFF in all of the experiments shown within the scope of this study can be assumed to be 1.67 times higher than the reported yields.

In a subsequent step, the process was analyzed at semi-preparative scale at higher volumes, using a stepwise scale up of the reaction was performed in 50% v/v H_2O. The reactions were performed on three different scales, with particular attention to keeping the same headspace-to-liquid ratio in the reaction vessels (9:1) (Table 2).

The results show comparable yields of DFF, which also resulted higher than the previously obtained one of 2.9 ± 0.4 % (3 mL liquid volume, 30 mL reaction volume, 0.250 g of immobilized biocatalyst), under the same conditions. These reactions were performed in vessels of a more optimal shape, which allowed for more favorable mixing. In addition, using different sized reaction vessels of identical shape vastly contributed to the high reproducibility of the reaction, which is expected to continue in higher scales as well.

Biocatalyst stability

Subsequently, the stability of the catalyst under real process conditions was investigated by performing the oxidation reaction five times using the same immobilized biocatalyst batch. The assessment was performed in 50% v/v H_2O as the optimal biphasic system, as well as in neat EtOAc and 100% buffer for comparison. In all three media conditions, the yield obtained in the second batch amounted to ≤ 50% of the one observed in the first batch (Figure 3). A reason for this may be the previously reported adsorption of DFF onto the carrier, with particular prominence in the buffer. The adsorption might occlude some of the enzyme molecules on the surface of the carrier, thus lowering the overall activity of the catalyst, as observed in other catalysts.[45] As stated above, the adsorption is most likely favored in buffer and is assumed to occur rather quickly. This could also explain the trend that with an increase in the amount of EtOAc in the reaction medium, a smaller difference in obtained yield between batches number 2 to number 5 was observed. GalOx-ECR8215F (epoxy carrier) could then be recycled four times, with a 44% loss of product formation in neat EtOAc, and 80% and 89% in 50% v/v H_2O and buffer after five batches, respectively. In any case, it must be noted that other aspects related to the reaction media may also play a role in the instability of the immobilized derivative, such as lack of minimal water in the medium in the case of

Table 2. Oxidation of 50 mM HMF to DFF in 50% v/v H_2O-EtOAc using GalOx-ECR8215F (epoxy carrier). The reaction was performed at 25 °C and 170 rpm for 72 hours, while keeping the headspace volume at a 9:1 ratio to the liquid volume. All reactions were performed in biological triplicates and subsequently analyzed using HPLC.

Total reaction volume (mL)	Total reactor volume (mL)	Biocatalyst amount (g)	DFF yield (%)
10	100	0.833	4.6 ± 0.1
25	250	2.083	4.8 ± 0.3
50	500	4.167	4.8 ± 0.1

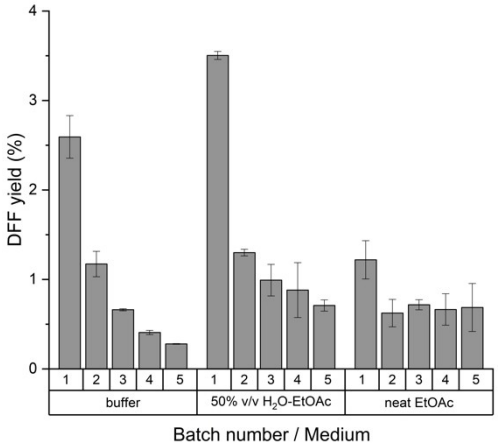

Figure 3. Yield of DFF after using the same batch of galactose oxidase immobilized on Purolite® Lifetech™ ECR8215F (epoxy carrier) in five consecutive 72-hour reaction cycles in 50% v/v H_2O EtOAc, buffer, and neat EtOAc. Reaction conditions: 0.250 g GalOx-ECR8215F, 50 mM HMF, 3 mL total liquid reaction volume in 30 mL reaction vials, 25 °C, and 150 rpm. After each 72 hours, the biocatalyst was washed in buffer and introduced to a new batch of substrate. All reactions were performed in biological triplicates and subsequently analyzed using HPLC.

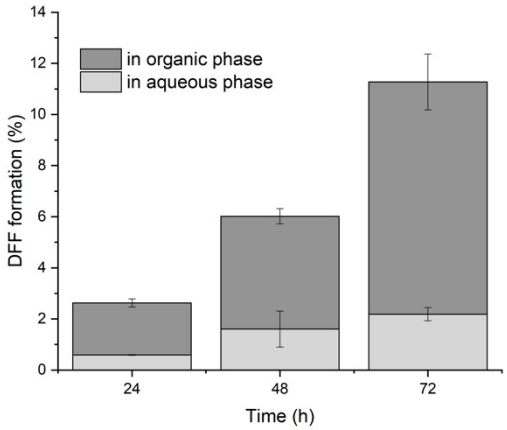

Figure 4. Yield of DFF after replacing the catalyst with a fresh batch every 24 hours, for a total reaction time of 72 h. Reaction conditions: 0.250 g GalOx-ECR8215F (epoxy), 50 mM HMF, 3 mL total liquid reaction volume (50% v/v EtOAc:Buffer) in 30 mL reaction vials, 25 °C, and 150 rpm. All reactions were performed in biological triplicates and subsequently analyzed using HPLC.

EtOAc, which, in turn, retrieves the necessary water molecules from the enzyme scaffold.

From the obtained results it can be concluded that an aqueous-ethyl acetate biphasic medium can be a promising reaction system for the GalOx-catalyzed enzymatic oxidation of HMF to DFF, while also being a potential biorefinery-like media. Thus, this connects some of the expected effluents in a biorefinery (such as HMF in ethyl acetate with traces of water) with enzymes able to perform selective biotransformations in such crude systems, eliminating the need of often costly purification or water removal steps to generate inert media necessary for more delicate catalysts. In that respect, biocatalysis has a tremendous potential to deliver "media-agnostic" enzymes that can properly and selectively work in these media.[9]

However, the obtained results show that some limitations still exist in the intended approach – such as biocatalyst stability, which could lead to the low yields observed in the reactions. To further investigate this, the reaction was set up in 50% v/v H_2O, and the biocatalyst was replaced every 24 hours by a freshly immobilized batch. Yields were measured in both phases to determine the distribution of DFF along the reaction (Figure 4).

As observed, the DFF yields increased linearly with each catalyst replacement. The first 72 hours of reaction time show that the amount of DFF in the EtOAc phase doubles every 24 hours, under the non-optimized conditions. Between the two phases combined, a total DFF yield of 11.3 ± 1.1 % was reached after 72 hours, compared to 2.9 ± 0.4 % obtained without biocatalyst replacement. Therefore, it can be concluded that the immobilized biocatalyst itself, albeit robust enough to perform reactions in the biorefinery-like media, is eventually deactivated. However, it must be noted that this strategy can be improved from several standpoints. Firstly, and most importantly, the use of genetically designed GalOx variants better adapted to the biorefinery-like media would undoubtedly lead to much higher catalytic efficiency and stability. [40] Moreover, the combination of GalOx with horseradish peroxidase and catalase may also increase the DFF yield (albeit increasing process costs too). [46] Finally, the reactor setup can be subject to consideration as well, by optimizing the oxygen availability and reagents mixing.

Conclusions

Furan-based compounds are becoming an increasingly important group of chemicals obtained in biorefineries. As they are produced upon acidic sugar dehydration in aqueous media, they are often extracted during downstream processing. Therefore, it is expected that in many biorefineries, furans will be delivered in crude organic media with traces of water, other chemicals, and remnants of biomass. Thus, it is important to establish catalytic systems that can perform reactions under those challenging conditions. While many chemocatalysts need purified media, often with a complete absence of water, enzymes can be adapted (or genetically designed) to perform highly selective reactions in crude media conditions.

Following these considerations, this paper has explored the use of immobilized galactose oxidase to conduct the selective oxidation of HMF to DFF. This synthesis is challenging, due to the inherent reactivity of both HMF and DFF, which leads to by-product formation and overoxidation. The application of enzymes, i.e. galactose oxidase, under mild conditions may give rise to the utilization of these chemicals in a cleaner process. Furthermore, the incorporation of ethyl acetate-based media with different amounts of water represents a realistic solvent mixture for biorefineries, and the immobilized GalOx can perform the expected reaction under those conditions. Thus, the covalent immobilization of GalOx onto Purolite® Lifetech™

epoxy-functionalized carriers proved advantageous for obtaining a catalyst with increased tolerance towards EtOAc. ECR8215F (epoxy) was the optimal carrier choice, with the highest activity yield and exceptional storage stability over 28 days.

The immobilization of GalOx puts forward the opportunity to simplify downstream processing and reuse the biocatalyst, which would be pivotal in biorefineries, where low-price large-volume commodity chemicals are expected. Nevertheless, this paper has also shown some important limitations to the approach, and further steps need to be taken to improve both the immobilization strategy and the reaction setup. Herein, aiming for higher protein loadings and optimization of the immobilization process (in terms of time, buffer pH, and molarity) could lead to higher immobilization and activity yields. It is also worth mentioning that HMF is a non-natural substrate for wild type GalOx used here, and that the use of a variant such as $M_{3\text{-}5}$ is expected to result in higher DFF yields. [40] On the other hand, transferring the reaction from closed vials to bioreactors would enable improved substrate mixing and therefore aeration, which would tip the balance towards the product side. Reactor configurations such as rotating bed reactors allow for better mixing without any detrimental effects on the enzyme, as well as provide easy catalyst removal and recycling. [47] Considering the ever-increasing demand for sustainable production, a biocatalyst that can directly oxidize HMF in the presence of organic solvents poses an invaluable asset for valorizing biorefinery waste, as well as establishing high-throughput production of DFF and other furan-based chemicals.

Experimental Section

Materials

The Lifetech™ enzyme carriers were generously provided by Purolite Ltd. (United Kingdom). Horseradish peroxidase and the Bradford assay solution were purchased from PanReac AppliChem ITW Reagents (Germany). Albumin bovine fraction V was purchased from Serva (Germany). 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). 2,5-diformylfuran was purchased from TCI Chemicals (Japan). All other chemicals were purchased from Sigma-Aldrich (Germany) and used as received.

Enzyme immobilization procedure

Galactose oxidase (GalOx) was immobilized on the amino- and epoxy-functionalized Lifetech™ carriers according to the instructions provided by the manufacturer. [48] Before immobilization, the carrier was washed in 0.1 M NaPi buffer pH 7.4 at a 1:1 (w:v) ratio for 5 minutes, and the liquid was removed by vacuum filtration. In the case of amino-functionalized carriers, the carriers were activated by incubating them in a 2% glutaraldehyde solution in a 1:4 (w:v) ratio for 1 hour, and subsequently washed again. The washed carrier was then mixed with a 2 mg mL^{-1} enzyme solution for 18 hours at room temperature and 60 rpm. Upon this, the mixture was vacuum filtered, and washed twice as previously described. The immobilized enzyme was stored at 4 °C. The collected supernatants, the initial enzyme solution, and the immobilized enzyme were analyzed sing the modified ABTS-HRP assay[49] and the Bradford assay[50] in order to obtain the activity measurements and protein concentrations necessary for calculating the immobilization parameters (equations available in SI).

ABTS-HRP activity assay

Activity measurements were performed using the modified ABTS-HRP spectrophotometric assay. [49] The assay is based on the presence of hydrogen peroxide (H_2O_2) in the reaction solution, originating from the oxygen-aided oxidation of D-galactose to D-galacto-hexodialdose by galactose oxidase. Horseradish peroxidase utilizes the H_2O_2 to oxidize ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to its cation radical, which causes an absorbance shift measured at 405 nm and 25 °C (Figure S3). Under these conditions, the molar extinction coefficient of ABTS was previously determined to be 30.9 L×min×mmol^{-1}. One unit of GalOx activity can be defined as the amount of enzyme necessary for the consumption of 1 μmol of O_2 per minute, or the oxidation of 2 μmol of ABTS per minute.[49]

The assay solution was prepared by combining 1 mM ABTS, 100 mM D-galactose, and 2 U mL^{-1} HRP in 100 mM NaPi buffer pH 7.4. The activity in the liquid enzyme preparations, *i.e.* free enzyme solution and the supernatants obtained during the enzyme immobilization procedure, was measured by adding 10 μL of the enzyme solution to 990 μL of the assay mixture, and continuously measuring the change in absorbance for 1 min. In the case of the immobilized enzyme, 5 mg of the catalyst were added to 10 mL of the assay solution under constant stirring in a round-bottom thermostated vessel, and single absorbance measurements were performed on five 1 mL samples taken at 20-second intervals. All activity measurements were performed in triplicates.

Bradford assay

Protein quantification was performed using the Bradford assay protocol for low protein quantities (1–25 μg mL^{-1}), modified to accommodate a microplate reader.[50] 150 μL of the sample and 150 μL of Bradford's reagent were added to each well. The microplate was gently mixed on a shaker for 30 seconds, and then left to incubate for 10 minutes. Absorbance was measured at 595 nm using the Tecan Infinite® M200 PRO plate reader (Austria). A standard curve was previously constructed using Albumin bovine fraction V, pH 7.0. The protein amount was determined in all three obtained filtrates, as well as the initial free enzyme solution.

HPLC analysis

HPLC analysis was performed on an Agilent Technologies (Germany) 1260 Infinity II high-performance liquid chromatography system with a diode-array detector (DAD) detector using a Rezex™ column (ROA, organic acid H + 8%, 300×7.8 mm) from Phenomenex (Germany). Isocratic elution was performed using 0.005 N H_2SO_4 in Milli-Q at a flow rate of 0.75 mL min^{-1} and 50 °C, with a total runtime of 42 minutes.[51] The sample injection volume was 10 μL, and HMF and DFF were detected at a wavelength of 280.4 nm. The chromatograms of the HMF and DFF standards are shown in Figure S4, along with a chromatogram of a representative reaction in Figure S5.

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Conflict of Interests

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.

Keywords: biocatalysis · enzyme immobilization · galactose oxidase · 5-hydroxymethylfurfural (HMF) · biphasic systems

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