


Review

# Whole Cell Biocatalysis of 5-Hydroxymethylfurfural for Sustainable Biorefineries

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**Abstract:** The implementation of cost-effective and sustainable biorefineries to substitute the petroleum-based economy is dependent on coupling the production of bioenergy with high-value chemicals. For this purpose, the US Department of Energy identified a group of key target compounds to be produced from renewable biomass. Among them, 5-hydroxymethylfurfural (HMF) can be obtained by dehydration of the hexoses present in biomass and is an extremely versatile molecule that can be further converted into a wide range of higher value compounds. HMF derivatives include 2,5-bis(hydroxymethyl)furan (BHMF), 5-hydroxymethyl-furan-2-carboxylic acid (HMFCFA), 2,5-diformylfuran (DFF), 5-formyl-2-furancarboxylic acid (FFCA) and 2,5-furandicarboxylic acid (FDCA), all presenting valuable applications, in polymers, bioplastics and pharmaceuticals. Biocatalysis conversion of HMF into its derivatives emerges as a green alternative, taking into account the high selectivity of enzymes and the mild reaction conditions used. Considering these factors, this work reviews the use of microorganisms as whole-cell biocatalysts for the production of HMF derivatives. In the last years, a large number of whole-cell biocatalysts have been discovered and developed for HMF conversion into BHMF, FDCA and HMFCFA, however there are no reports on microbial production of DFF and FFCA. While the production of BHMF and HMFCFA mainly relies on wild type microorganisms, FDCA production, which requires multiple bioconversion steps from HMF, is strongly dependent on genetic engineering strategies. Together, the information gathered supports the possibility for the development of cell factories to produce high-value compounds, envisioning economical viable biorefineries.

**Keywords:** whole-cell biocatalysts; 5-hydroxymethylfurfural derivatives; bioconversion; biorefinery



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## 1. Introduction

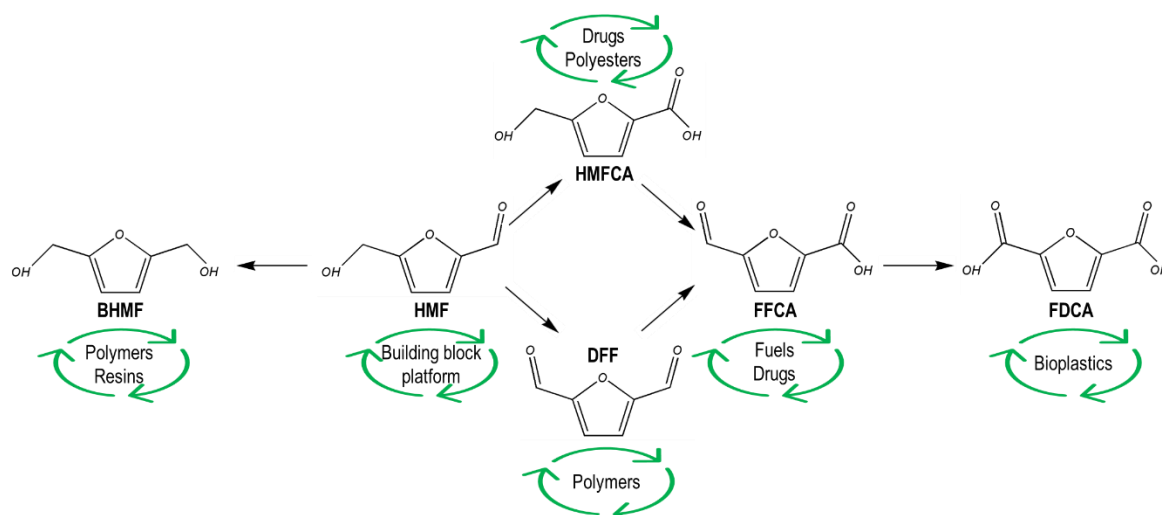
The world faces the progressive depletion of its fossil fuels reserves, with excessive consumption leading to an increase in the emission of greenhouse gases and in climate changes promoted by global warming. These factors, coupled with the growing demand for energy for transportation, heating, industrial processes, among others, highlight the necessity to transition from a petroleum-based towards a bio-based economy. This bioeconomy is based on the biorefinery concept, defined as “the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)” [1].

Biorefineries have been mostly conceptualized around energy and biofuels. More recently, process integration and conversion of all fractions of the biomasses into value-added products has been recognized as key elements for a profitable biorefinery operation. This encompasses the two main goals of a biorefinery: (1) the energetic goal, focusing on the replacement of petroleum by renewable carbons for fuel production, and (2) the economic goal, directing efforts towards the efficient production of biobased chemicals providing the financial incentive to maintain and expand the biorefining industry.

Aiming at the economic viability of biorefineries, Bozell and Petersen suggested that the manufacture of low-value biofuels should be supplemented with the production of high-value biobased chemicals [2]. They also presented an updated list of biobased product opportunities from renewable carbohydrates, based on the one published by the US Department of Energy in 2004 [3], which consists of ethanol, furans (5-hydroxymethylfurfural, furfural and FDCA), glycerol and derivatives, biohydrocarbons (isoprene), organic acids (lactic, succinic, levulinic and 3-hydroxypropanoic acids) and sugar alcohols (xylitol and sorbitol). This selection was made considering the type of raw material, production costs, sales prices and availability of processes and technologies. Considering that the conversion of renewable carbon into chemicals is the more challenging and least developed step of all biorefinery operations, there is an urgent necessity to identify and develop microbial catalysts for an efficient production of biofuels (e.g., bioethanol), as well as other top value compounds from renewable biomass.

## 2. 5-Hydroxymethylfurfural Derivatives

The versatile composition of 5-hydroxymethylfurfural (HMF)—consisting of an aromatic furan ring and reactive functional groups (aldehyde and alcohol groups)—makes it an attractive building block platform, as it can be transformed into higher value derivative compounds, having applications in several areas such as plastic, pharmaceutical, fragrance and textile industries (Figure 1). Accordingly, the global market of HMF is expected to reach \$61 million by 2024 [4]. HMF is commonly generated during pretreatment of lignocellulosic biomass and is usually regarded as a microbial inhibitor [5]. It derives from the dehydration of hexoses (such as the cellulose-derived glucose), and its accumulation depends on the biomass used and the type and severity of pretreatment and hydrolysis applied. As its value has been receiving growing attention, several studies focused on optimizing pretreatment methods to increase HMF accumulation from different biomasses by using different reaction media and/or catalysts (e.g., solid acid catalysts, Lewis acids, Brønsted acids, ionic liquids, salts, deep eutectic solvents and biphasic systems) as well as alternative heating methods (e.g., ultrasound and microwave) [6–10].



**Figure 1.** Reduction and oxidation derivatives of HMF and corresponding application. BHMf: 2,5-bis(hydroxymethyl)furan. HMF: 5-hydroxymethylfurfural. HMFCA: 5-hydroxymethyl-furan-2-carboxylic acid. DFF: 2,5-diformylfuran. FFCA: 5-formyl-2-furancarboxylic acid. FDCA: 2,5-furandicarboxylic acid.

Accordingly, increased importance has been given to the conversion of HMF into higher value derivatives [11,12]. Among these, 2,5-furandicarboxylic acid (FDCA) was also identified as one of the top products to be obtained from biomass [2], and its worldwide market is expected to reach \$850 million by 2025 [13]. Its main application is the poly-

merization with ethylene glycol to produce polyethylene furanoate (PEF), a substitute to petroleum-derived polyethylene terephthalate (PET) plastic. In fact, the European Union's Horizon 2020 funded a consortium of eleven companies named to establish an innovative biobased production of FDCA and PEF (<https://reference.eu/> (accessed on 9 December 2021)). FDCA is obtained by oxidation of HMF, and some microorganisms such as *Acinetobacter oleivorans*, *Aspergillus flavus* and *Burkholderia cepacian* [14–16] can produce it natively. Nevertheless, chemical processes for its production are applied in most industries, with Corbion being a pioneer in using microbial biocatalyst to produce FDCA from HMF. In fact, while chemocatalytic processes are still the main transformation strategy used, their application requires the use of metals (mostly noble) as catalysts and harsh operating conditions, generating hazardous by-products [17]. Considering these, and aiming at sustainable development, biocatalysis has been receiving increased attention due to its milder conditions, higher selectivity and environmental friendliness [18]. Moreover, considering these factors, the use of whole-cell biocatalysts presents advantages over enzyme biocatalysis, such as regeneration of co-factors and ease of catalyst recycling [19,20].

The chemistry of HMF, containing aldehyde and alcohol functional groups, makes it highly reactive towards oxidation–reduction and other reactions. Besides the already mentioned FDCA, other derivatives with valuable applications can be obtained from HMF, such as 2,5-bis(hydroxymethyl)furan (BHMF), 5-hydroxymethyl-furan-2-carboxylic acid (HMFCA), 2,5-diformylfuran (DFF) and 5-formyl-2-furancarboxylic acid (FFCA) (Figure 1). Considering these, and to contribute to the replacement of chemical approaches and development of sustainable biorefineries, this review focuses on the reported microorganisms used as whole-cell biocatalysts for the production of these HMF derivatives. For that, each HMF derivative will be approached, including the most relevant results obtained.

### 2.1. 2,5-Bis(Hydroxymethyl)Furan (BHMF)

BHMF has a potential application in the synthesis of polymers, resins and ethers [18]. BHMF was first reported by Liu et al. [21] as the reduction product of HMF by *S. cerevisiae*. More recently, fungi, yeast and bacteria strains have been described as biocatalysts for its production from HMF (Table 1). The bacteria *Paraburkholderia azotifigens* F18 was found to produce 36.9 mM of BHMF from HMF, with a 92% yield, under anaerobic conditions [22]. The yeast *Meyerozyma guilliermondii* SC1103 was identified as a BHMF producer [23], and its conversion abilities were further improved by cell acclimatization and immobilization in calcium alginate beads, reaching BHMF titers of 181 mM, with 85% and a productivity of 25.8 mM/h [24]. Furthermore, in a process with cell recycling the yeast maintained good catalytic activities during four runs with yields between 81% and 88% and a cell viability of up to 70% after the 4th run [24]. An *Escherichia coli* strain was modified to harbor a NADH-dependent reductase from *Candida magnoliae* and produced 181 mM of BHMF with 91% yield [25]. In another genetic engineering strategy, a *S. cerevisiae* strain harboring an aryl alcohol dehydrogenase from *M. guilliermondii* reached a BHMF titer of 345 mM with a productivity of 15 mM/h, presenting however a low yield of ~77% [26]. Moreover, in this work, an inexpensive corncob hydrolysate was described as a promising alternative to glucose as co-substrate for the biocatalysis. The fungus *Aureobasidium subglaciale* F134 was identified for the production of BHMF, reaching the highest reported productivity of 28.7 mM/h with a fed-batch process [27]. Another fungus, *Fusarium striatum*, was reported to produce 145 mM of BHMF with high yield of 97% [28]. The bacterium *Burkholderia contaminans* NJPI-15 reached the highest BHMF titer of 656 mM in a fed-batch with 94% yield [29]. More recently, the highest BHMF yield of 99.65% was reported using a *Kluyveromyces marxianus* yeast strain isolated from cocoa fermentation, which reached an BHMF titer of 55.3 mM in a batch process [30].

**Table 1.** Whole-cell biocatalysts reported for the conversion of 5-hydroxymethylfurfural (HMF) into 2,5-bis(hydroxymethyl)furan (BHMF). Yield is presented as percentage of the theoretical molar yield. OD: optical density. CDW: cell dry weight. CTAB: cetyltrimethyl ammonium bromide. YPD: Yeast extract peptone dextrose.

Strain	Conditions	Cell Inoculum	Medium	Initial [HMF] (mM)	Process	Final [BHMF] (mM)	Yield (%)	Productivity (mM/h)	Ref.
<i>Paraburkholderia azotifigens</i> F18	30 °C, pH 7, 150 rpm	67 g/L wet cells	100 mM phosphate buffer with 40 mM glucose	40 mM	Batch, anaerobic	36.9	92	-	[22]
<i>Meyerozyma guilliermondii</i> SC1103	35 °C, pH 7.2, 200 rpm	20 g/L wet cells	100 mM phosphate buffer with glucose	100	Batch	86	86	7.17	[23]
				50	Fed-batch: ~217 mM total HMF	191	~88	7.80	
<i>Meyerozyma guilliermondii</i> SC1103	35 °C, pH 8, 200 rpm	20 g/L wet cells	100 mM Tris-HCl buffer with glucose	213	Batch. Cell acclimatization and immobilization calcium alginate beads	181	85	25.8	[24]
<i>Escherichia coli</i> CCZU-K14 harboring NADH-dependent reductase from <i>Candida magnoliae</i>	30 °C, pH 6.5, 160 rpm	100 g/L wet cells	Glucose, xylose, l-glutamic acid, Mg <sup>2+</sup> , β-cyclodextrin, and CTAB	200	Batch.	181	91	2.51	[25]
<i>Saccharomyces cerevisiae</i> harboring an aryl alcohol dehydrogenase from <i>M. guilliermondii</i>	30 °C, pH 8, 200 rpm	60 g/L wet cells	100 mM Tris-HCl buffer and glucose	250	Batch	235	94	9.79	[26]
				150	Fed-batch: ~450 mM total HMF	345	~77	15.0	
<i>Aureobasidium subglaciale</i> F134	30 °C, pH 7, 850 rpm	200 g/L wet cells	100 mM phosphate buffer with Zn <sup>2+</sup> ion	180	Batch	148	82	16.4	[27]
				100	Fed-batch: ~500 mM total HMF	430	86	28.7	
<i>Fusarium striatum</i>	28 °C, pH 7, 160 rpm	4.0 × 10 <sup>6</sup> spores/mL	Malt extract media with glucose	75	Fed-batch: 150 mM total HMF	145	97	2.42	[28]
<i>Burkholderia contaminans</i> NJPI-15	35 °C, pH 7, 180 rpm	20 g/L wet cells	50 mM PBS with glutamine, sucrose and Mn <sup>2+</sup>	100	Batch	95	95	nd	[29]
				125	Fed-batch: 700 mM total HMF	656	94	13.7	
<i>Kluyveromyces marxianus</i>	37 °C, 150 rpm	100 g/L wet cells	YPD medium (111 mM glucose)	55.5	Batch	55.3	100	4.61	[30]

## 2.2. 2,5-Furandicarboxylic Acid (FDCA)

As already mentioned, the main application of FDCA is as a substitute of terephthalic acid, which can be used to synthesize several polyesters, with PEF being the most studied [31]. In addition, it can also be used as a building block in the production of medicines and polyamides [32].

The first microbial biocatalyst reported for FDCA production from HMF was a *Pseudomonas putida* S12 strain modified to express the *hmfH* gene from *Cupriavidus basilensis* (encoding an HMF/furfural oxidoreductase), which resulted in the production of 193 mM with 97% yield in a fed-batch strategy [33]. More recently, the *hmfo* from *Methylovorus* sp. strain MP688, encoding a HMF oxidase, was expressed in *P. putida* S12, which resulted in the production of 545 mM of FDCA in a fed-batch [34]. *P. putida* S12 was also modified by CRISPR for the development of more stable strains [35]. With this technique, this bacterium was modified to express *hmfH* and *HMFT1* (encoding a HMF transporter) genes from *C. basilensis*, which allowed the production of 196 mM of FDCA with 78% yield. In a consortium approach, a *Synechococcus elongatus* strain engineered to export sucrose via CO<sub>2</sub> fixation was used as carbon source for a *P. putida* S12 strain modified for sucrose consumption and improved FDCA production [36]. Despite being innovative and having promise for reducing feedstocks costs within a biorefinery, this strategy only resulted in the production of ~4.6 mM of FDCA. Tan and collaborators [37] reported a whole-cell cascade, where an engineered *E. coli* was coupled with a wild type *P. putida* in a stepwise reaction, where HMF would be oxidized into DFF by *E. coli*, which would be further oxidized into FDCA by addition of a *P. putida* strain. This allowed the conversion of 100 mM of HMF with a FDCA yield higher than 99%, however the reaction required the addition of the commercial enzymes horseradish peroxidase (as an enzyme activator) and catalase (to decompose H<sub>2</sub>O<sub>2</sub>) [37].

Hossain et al. [38] isolated a *Raoultella ornithinolytica* BF60 strain capable of converting HMF into FDCA with 51.0% yield. This bacterium was modified to prevent FDCA degradation to furoic acid and HMF reduction into BHMF, and to improve HMF oxidation into FDCA by overexpression of the gene encoding aldehyde dehydrogenase 1, which increased FDCA yield to 89%, corresponding to a titer of 89.1 mM [38]. The same research group also modified the *R. ornithinolytica* BF60 strain to express the genes *hmfo* from *Methylovorus* sp. strain MP688, encoding HMF oxidase, and *hmfH* from *C. basilensis* HMF14, encoding HMF/furfural oxidoreductase, which improved the FDCA titer to 93.6 mM and yield to 94% [39]. In a different study using *Raoultella ornithinolytica* BF60 as host, combinatorial synthetic pathway fine-tuning of *hmfo* and *hmfH*, as well as deletion of five genes associated with HMF reduction, resulted in increased FDCA titers of 221.5 mM with an 89% yield in a fed-batch strategy [40]. In another fed-batch approach, FDCA production was further improved to titers of 264.7 mM with 96% yield by using a modified *R. ornithinolytica* BF60 strain with deletion of two genes responsible for the reduction of HMF into BHMF and overexpression of *AldH*, encoding an enzyme responsible for the oxidation of FFCA into FDCA [41].

Using the bacteria *E. coli* as cell host, FDCA production was attained by modification with *hmfH* from *C. basilensis* HMF14 and vanillin dehydrogenase (VDH1) from *Comamonas testosteroni* SC1588, resulting in the production of 144 mM of FDCA with a yield of 96% [42]. These values are in line with the ones obtained with modified *P. putida* S12 and *R. ornithinolytica* BF60 (Table 2).

**Table 2.** Whole-cell biocatalysts reported for the conversion of 5-hydroxymethylfurfural (HMF) into 2,5-furandicarboxylic acid (FDCA). Yield is presented as percentage of the theoretical molar yield. OD: optical density. CDW: cell dry weight.

Strain	Modification	Conditions	Cell Inoculum	Medium	Initial [HMF] (mM)	Process	Final [FDCA] (mM)	Yield (%)	Productivity (mM/h)	Ref.
<i>Burkholderia cepacia</i> H-2	None	28 °C, pH 7, 120 rpm	OD <sub>600</sub> = 0.1	Mineral salt media	12.8	Batch	8.17	64	0.340	[16]
			OD <sub>600</sub> = 0.25	Undiluted algal acid hydrolysate (supplemented with HMF)			6.34	50	0.264	
<i>Methylobacterium radiotolerans</i> G-2	None	26 °C, pH 7, 120 rpm	OD <sub>600</sub> = 0.1	Mineral salt media	6.41		3.29	51	0.137	[43]
			OD <sub>600</sub> = 0.25	Two-fold diluted algal acid hydrolysate (supplemented with HMF)	nd		2.94	nd	0.0817	
<i>Pseudomonas putida</i> S12	<i>hmfH</i> from <i>Cupriavidus basilensis</i> HMF14, encoding HMF/furfural oxidoreductase	30 °C, pH 7, 150 rpm	0.2 g/L CDW	Mineral medium with glycerol	0	Fed-batch: 188 mmol total HMF	193	97	1.26	[33]
<i>P. putida</i> S12	<i>hmfo</i> from <i>Methylovorus</i> sp. strain MP688 encoding HMF oxidase	30 °C, pH 8	Near 0 g/L CDW	Mineral media with glycerol and MgCl <sub>2</sub>	0	Fed-batch: total HMF not defined	545	nd	7.57	[34]
<i>P. putida</i> S12	<i>hmfH</i> (encoding HMF/furfural oxidoreductase) and <i>HMF11</i> (encoding a HMF transporter) from <i>C. basilensis</i> HMF14	30 °C, pH 7, 180 rpm	OD <sub>600</sub> = 20	Mineral media with MnO <sub>2</sub> and CaCO <sub>3</sub>	250	Batch	196	78	8.17	[35]
<i>Synechococcus elongatus</i> PCC7942 and <i>P. putida</i> S12	Sucrose symporter ( <i>CscB</i> ) of <i>Escherichia coli</i> in <i>S. elongatus</i> . Sucrose-6-phosphate hydrolase ( <i>CscA</i> ) from <i>E. coli</i> W and <i>hmfH</i> from <i>C. basilensis</i> HMF14 in <i>P. putida</i>	30 °C, pH 7.3	<i>S. elongatus</i> (OD <sub>750</sub> ~0.8) and <i>P. putida</i> (OD <sub>600</sub> of 1)	30 mM HEPES-NaOH	~1	Fed-batch: total HMF > 6 mM	~4.6 mM	nd	Nd	[36]
<i>Escherichia coli</i> M and <i>P. putida</i> KT2440	Galactose oxidase (GOase M <sub>3-5</sub> ) in <i>E. coli</i>	37 °C, pH 7, 200 rpm	<i>E. coli</i> M (8.5 g/L DCW) and <i>P. putida</i> (6 g/L DCW)	100 mM sodium phosphate buffer, HRP, catalase and CaCO <sub>3</sub>	100	Batch. Stepwise addition of <i>E. coli</i> followed by <i>P. putida</i>	>99	>99	>15.6	[37]
<i>Raoultella ornithinolytica</i> BF60	None	30 °C, pH 8, 150 rpm	45 g/L wet cells	50 mM phosphate buffer	100	Batch	50.6	51	0.294	[38]
	Mutation of <i>dcaD</i> , encoding dicarboxylic acid decarboxylase. Mutation of <i>aldR</i> , encoding aldehyde reductase. Overexpression of the gene encoding aldehyde dehydrogenase 1.						89.0	89	0.517	

Table 2. Cont.

Strain	Modification	Conditions	Cell Inoculum	Medium	Initial [HMF] (mM)	Process	Final [FDCA] (mM)	Yield (%)	Productivity (mM/h)	Ref.
<i>R. ornithinolytica</i> BF60	<i>hmfo</i> from <i>Methylovorus</i> sp. strain MP688 encoding HMF oxidase and <i>hmfH</i> from <i>C. basilensis</i> HMF14 encoding for HMF/Furfural oxidoreductase	30 °C, pH 8, 220 rpm	45 g/L wet cells	50 mM phosphate buffer	100	Batch	93.6	94	0.979	[39]
<i>R. ornithinolytica</i> BF60	Combinatorial synthetic pathway fine-tuning of <i>hmfo</i> and <i>hmfH</i> . Deletion of <i>aldR</i> , <i>dkgA</i> , <i>akR</i> , <i>AdhP1</i> and <i>AdhP2</i> , involved in the reduction of HMF to BHMF.	30 °C, pH 8, 220 rpm	OD <sub>600</sub> = 100	50 mM phosphate buffer with CaCO <sub>3</sub>	100	Pulse addition: 250 mM total HMF	222	89	1.66	[40]
<i>R. ornithinolytica</i> BF60	Deletion of <i>adhP3</i> and <i>alkR</i> , involved in the reduction of HMF to BHMF. Overexpression of <i>aldH</i> , responsible for the oxidation of FFCA into FDCA	30 °C, pH 8	OD <sub>600</sub> = 100	50 mM phosphate buffer with CaCO <sub>3</sub>	50	Pulse addition: 275 mM total HMF	265	96	1.84	[41]
<i>E. coli</i>	<i>hmfH</i> from <i>C. basilensis</i> HMF14 and vanillin dehydrogenase (VDH1) from <i>Comamonas testosteroni</i> SC1588	30 °C, pH 7, 150 rpm	150 g/L wet cells	200 mM phosphate buffer	150	Batch	144	96	2	[42]

Several other microorganisms have been described to convert HMF into FDCA, such as the bacteria *Burkholderia cepacia* H-2 [16], *Methylobacterium radiotolerans* G-2 [43], *Enterobacter* sp. [44], *Acinetobacter oleivorans* S27 [14], *Acinetobacter calcoaceticus* NL14 [45] and *Bacillus toyonensis* [46] and the filamentous fungi *Aspergillus flavus* APLS-1 [15], *Aspergillus niger* [47] and *Trichoderma reesei* [47]. Nonetheless, FDCA titers obtained with these biocatalysts were low (<8.2 mM), highlighting the importance of genetic engineering approaches for the production of this HMF derivative. Despite the low values obtained, these reports broaden the pool of possible hosts for further modifications for improved FDCA production. Furthermore, note that two of these species, *Burkholderia cepacia* H-2 and *Methylobacterium radiotolerans* G-2, were used as biocatalysts in undiluted and 2-fold diluted macroalgae acid hydrolysate supplemented with HMF, showing their capacity to maintain HMF conversion even in the presence of other microbial inhibitors such as furfural and weak acids [43]. Furthermore, the same group immobilized *B. cepacia* H-2 in calcium-alginate beads and evaluated its capacity to produce FDCA from HMF in the presence of lignocellulosic-derived inhibitors [48]. The immobilized strain was able to produce FDCA in the presence of up to 5 g/L formic acid, 4.39 g/L acetic acid, 5 g/L levulinic acid, 0.5 g/L furfural and 0.5 g/L phenol. This is relevant because the severity of the conditions needed to generate high HMF quantities from biomass would also result in high accumulation of other inhibitory compounds. While the Corbion Company is known to produce FDCA from HMF with microbial catalysts, there is not enough information regarding the HMF source, and to the extent of our knowledge, there is still no reports of FDCA production by whole-cell biocatalysts from biomass-derived HMF.

### 2.3. 5-Hydroxymethyl-Furan-2-Carboxylic Acid (HMFCa)

HMFCa is an antitumor agent [49] and interleukin inhibitor [50], and it can be also used as an intermediate in the preparation of polyesters [51]. Early reports on HMFCa microbial production refer to it as a by-product or intermediate and are exploratory regarding HMF oxidation/degradation products [33,52,53]. As an exception, in 2004 Mitsukura and collaborators [54] focused on obtaining carboxylic acids from aromatic aldehydes and reported a *Serratia liquefaciens* LF14 strain capable of attaining 168 mM of HMFCa from HMF with 97% yield [54]. More recently, great effort has been put into HMFCa and finding whole-cell biocatalysts capable of efficiently produce it from HMF (Table 3). Mostly, bacteria species have been described for this purpose, e.g., *Serratia* spp., *Comamonas testosteroni*, *Deinococcus wulumuqiensis*, *Pseudomonas* spp. and *Gluconobacter oxydans*. In a patent that focuses on fungal production of FDCA, the *S. cerevisiae* CEN.PK113-1A strain has been reported to produce 2.84 mM of HMFCa with a 93% yield [55]. Fed-batch strategies have been recurrently used to diminish the toxic effect of HMF on the cells, allowing for higher HMFCa titers and productivity, up to 809 mM and 25.6 mM/h, respectively (Table 3). In the case of *Comamonas testosteroni* SC1588, its catalytic performance (e.g., substrate tolerance and catalytic efficiency) was improved by a substrate adaptation strategy, reaching a HMFCa yield near 100% in 24 h from 200 mM of HMF [56].



**Table 3.** Whole-cell biocatalysts reported for the conversion of 5-hydroxymethylfurfural (HMF) into 5-hydroxymethyl-furan-2-carboxylic acid (HMFA). Yield is presented as a percentage of the theoretical molar yield. OD: optical density. CDW: cell dry weight.

Strain	Conditions	Cell Inoculum	Medium	Initial [HMF] (mM)	Process	Final [HMFA] (mM)	Yield (%)	Productivity (mM/h)	Ref.
<i>Serratia liquefaciens</i> LF14	30 °C, pH 7, 150 rpm	~12 g/L CDW	100 mM potassium buffer	nd	Fed-batch: 173 mM total HMF	168	97	nd	[54]
<i>Saccharomyces cerevisiae</i> CEN.PK113-1A	pH 7, 150 rpm	Nd	Mineral medium with glucose	3.05	Batch	2.84	93	nd	[55]
<i>C. testosteroni</i> SC1588	30 °C, pH 7, 160 rpm	70 g/L wet cells	200 mM phosphate buffer and histidine	200	Batch with substrate adaptation strategy	~200	~100	~8.3	[56]
<i>E. coli</i> modified with 3-succinylsemialdehyde-pyridine dehydrogenase from <i>C. testosteroni</i> SC1588	30 °C, pH 7, 160 rpm	50 g/L wet cells	200 mM phosphate buffer	50	Batch	47.5	95	9.5	[57]
				175		nd	>90	nd	[58]
				200		nd	<80	nd	
<i>E. coli</i> modified with vanillin dehydrogenase from <i>C. testosteroni</i> SC1588	30 °C, pH 7, 150 rpm	50 g/L wet cells	200 mM phosphate buffer	200	Batch	~184	~92	~15.3	[59]
				~50	Fed-batch 1st run: 333 mM total HMF	292	88	14.2	
<i>E. coli</i> modified with vanillin dehydrogenase from <i>C. testosteroni</i> SC1588 and NADH oxidase from <i>Lactobacillus brevis</i>	30 °C, pH 7, 150 rpm	50 g/L wet cells	200 mM phosphate buffer and NaHCO <sub>3</sub>	~50	Fed-batch 2nd run: >160 mM total HMF	149	nd	nd	[60]
				250	Batch	238	95	26.0	
<i>E. coli</i> modified with mutated <i>hmfO</i> from <i>Methylovorus</i> sp. strain MP688	30 °C, pH 7, 200 rpm	50 g/L wet cells	100 mM phosphate buffer	150	Batch	145	97	1.5	[61]
<i>Paraburkholderia azotifigens</i> F18 with deletion of genes encoding HMF oxidoreductase/oxidase	30 °C, pH 7, 200 rpm	133 g/L wet cells	Reaction buffer with glucose	150	Batch	147.5	98	3.07	[22]
<i>Serratia marcescens</i>	30 °C, pH 8, 200 rpm	OD = 0.05	Minimal salt medium	3 (crude HMF)	Fed-batch: total HMF not defined	5.56	28	0.278	[62]
			200 mM phosphate buffer	75.3	Batch	73.9	98	73.9	
<i>Pseudomonas putida</i> ATCC 47054	35 °C, pH 6, 200 rpm	8 g/L DCW	<i>G. amansii</i> hydrolysates	79.1 (biomass-derived)	Batch	78.0	99	44.6	[63]
			Soybean dreg hydrolysate, NaCl, corn cob residue with cellulase, and rhamnolipid; pH tuning	100	Fed-batch: 900 mM total HMF	809	90	13.0	
<i>Pseudomonas aeruginosa</i> PC-1	35 °C, pH 7, 180 rpm	5 g/L wet cells	Soybean dreg hydrolysate, NaCl, corn cob residue with cellulase, and rhamnolipid; pH tuning	100	Fed-batch: 900 mM total HMF	809	90	13.0	[64]
<i>Comamonas testosteroni</i> SC1588	30 °C, pH 7, 150 rpm	30 g/L wet cells	200 mM phosphate buffer with histidine and pH tuning	160	Batch with substrate adaptation strategy	157	98	4.36	[65]
				300	Batch	~270	~90	7.5	
<i>Deinococcus wulumuqiensis</i> R12	35 °C, pH 7	200 g/L wet cells	100 mM phosphate buffer	300	Batch	~270	~90	7.5	[66]
				150	Fed-batch: 601 mM total HMF	511	85	25.6	

Table 3. Cont.

Strain	Conditions	Cell Inoculum	Medium	Initial [HMF] (mM)	Process	Final [HMFA] (mM)	Yield (%)	Productivity (mM/h)	Ref.
<i>Pseudomonas putida</i> KT2440	35 °C, pH 6.5, 200 rpm	OD600 = 25	200 mM phosphate buffer and CaCO <sub>3</sub>	160	Batch	155	97	12.9	[67]
<i>Gluconobacter oxydans</i> DSM 50049		24 g/L DCW	sodium phosphate buffer and controlled pH	250	Batch	250	100	41.7	[68]
				250	Fed-batch: 345 mM total HMF	314	91	13.6	
<i>Pseudomonas aeruginosa</i> PC-1	30 °C, pH 7, 180 rpm	5 g/L wet cells	Sucrose, peptone and NaCl; pH tuning	100	Fed-batch: 800 mM total HMF	721	90	12.4	[69]

Differently from the microorganisms reported for FDCA production (Table 2), most of the whole-cell biocatalysts for HMFCa production require no genetic modification, but rather optimization of reaction conditions (e.g., temperature, pH and inoculum) to achieve high titers and yields. Despite the satisfactory values obtained with wild type strains, efforts have been made to genetically engineer bacteria to produce HMFCa. An *E. coli* strain modified with 3-succinoylsemialdehyde-pyridine dehydrogenase (SAPDH) from *C. testosteroni* SC1588 was able to produce HMFCa from 50 mM HMF with 95% yield [57]. Later, this strain was found to maintain high HMFCa yields even from higher HMF concentrations: ~90% from 175 mM HMF and almost 80% from 200 mM HMF [58]. A vanillin dehydrogenase from *C. testosteroni* SC1588 was also introduced in an *E. coli* strain by the same research group, resulting in the conversion of 200 mM HMF with 92% yield, which was further improved by a fed-batch strategy resulting in the synthesis of 292 mM of HMFCa [59]. Furthermore, these authors attempted a cell recycling strategy after the fed-batch, and despite the decrease in cell viability, 149 mM of HMFCa was produced in 18 h in the second run, maintaining the yields of the first run [59]. In a subsequent work of the same group, a cofactor-engineered *E. coli* with co-expression of vanillin dehydrogenase and NADH oxidase resulted in improved HMFCa titers and productivities [60]. The significant improvement in HMFCa productivity and HMF tolerance of these *E. coli* strains harboring *C. testosteroni* enzymes in comparison with the use of wild type *C. testosteroni* as a biocatalyst (Table 3) should be highlighted. Wang et al. [61] constructed a recombinant *E. coli* harboring a *hmfo* from *Methylovorus* sp. strain MP688 mutated for specificity for HMFCa rather than FDCA production, achieving HMFCa titers of 145 mM with 97% yield. Despite presenting higher HMFCa yield than the other recombinant *E. coli* strains, its productivity is significantly (up to 10 times) lower (Table 3). Surprisingly, in that work the authors did not explore the potential of the *E. coli* modified with the original *hmfo* for FDCA production, which presented yields of ~80%. The *Paraburkholderia azotifigens* F18 strain, capable of producing BHMF under anaerobic conditions, was found to accumulate HMFCa in more aerobic conditions, and with deletion of genes encoding HMF oxidoreductase/oxidase (to prevent oxidation of HMFCa to FDCA) it produced 147.5 mM of HMFCa with 98% yield [22].

The majority of the reports on whole-cell production of HMFCa use commercial HMF as substrate. Muñoz and collaborators [62] reported the bioconversion of crude HMF, obtained by dehydration of fructose, into HMFCa by the bacteria *Serratia marcescens*. The use of crude HMF, which more closely mimics real conditions and contains other by-products (such as furfural), severely hinders HMFCa yields and microbial tolerance [62], resulting in low HMFCa titers even with fed-batch strategies (Table 3). Liu and collaborators [63] produced HMF from the macroalgae *Gelidium amansii* with a thermal pretreatment with 2% (*w/w*) HCl at 120 °C for 40 min and identified *P. putida* ATCC 47054 as an efficient biocatalyst, which was capable of producing 78.0 mM of HMFCa from *G. amansii* hydrolysate with an 99% yield and the remarkable productivity of 44.6 mM/h. Differently from the results with crude HMF obtained from fructose, the HMFCa yield obtained from *G. amansii* hydrolysates was equal to the results with commercial HMF, with only HMFCa productivity being affected (Table 3). These results prove that the use of whole-cell biocatalysts for the conversion of biomass-derived HMF into higher value compounds is a promising strategy and should be further explored. Furthermore, the material cost of HMFCa production can be decreased to 11.47% of the traditional method by using corncob residue and soybean dreg as nutrient sources, which resulted in a fed-batch production of 809 mM HMFCa, from commercial HMF, with 90% yield and using a *Pseudomonas aeruginosa* PC-1 strain [64].

### 3. Conclusions

HMF is a building block platform with potential to be explored by microbial conversion. This review discusses the array of microorganisms used as whole-cell biocatalysts for the production of HMF derivatives. While there are no reports on microbial production of DFF and FFCA, several whole-cell biocatalysts have been discovered and developed

for HMF conversion into BHMF, FDCA and HMFCFA. With those, the use of fed-batch and pulse addition approaches resulted in the production of high compound titers, circumventing the toxic effect of HMF. The toxicity of the HMF derivatives should also be considered, and this can be lowered by using two-phase systems for an in situ extraction of the product, which also facilitates the downstream process. Regarding the optimization of process conditions, much of the focus is given to the optimal temperature and pH of biocatalysis. However, other parameters, such as aeration and presence of co-substrates, have been poorly explored despite being reported to play an important role on HMF conversion (e.g., favoring either the reduction or the oxidation of HMF). This review also shows that while the production of BHMF and HMFCFA mainly relies on wild type microorganisms, FDCA production, which requires more bioconversion steps from HMF, is strongly dependent on genetic engineering strategies. Genetic engineering strategies were also found to play an important role in preventing the accumulation of by-products (e.g., preventing further oxidation of HMFCFA), and should be further explored to increase the specificity of the process (e.g., to avoid BHMF accumulation when aiming at producing HMFCFA and FDCA, and vice versa). Furthermore, genetic modifications should also be used to develop more efficient whole-cell biocatalysts (e.g., following approaches already used in different hosts or expressing enzymes with reported activity for conversion of HMF into the target derivative). Furthermore, note that the vast majority of works discussed in this review report production of HMF derivatives using commercial HMF, where no other inhibitors are present. It is inevitable that the production of HMF from renewables biomasses would also result in the co-production of other inhibitory compounds, such as weak acids and furfural, which are known to affect the microbial conversion of HMF. In this sense, the success of using whole-cell biocatalysts for the production of HMF derivatives is also dependent on the evolution of the techniques used to produce HMF from renewable biomasses: envisioning HMF-enriched media with or without low concentrations of inhibitors and containing appropriate amounts of co-substrates (e.g., glucose for BHMF production). Alternatively, HMF purification techniques can be applied, but these will represent an increase in the cost of the process. Together, this information shows that the establishment of economically viable microbial biorefineries based on HMF derivatives is dependent not only on more efficient whole-cell biocatalysts, but also on the development of all the steps of the process, with important focus on the production of HMF from raw materials and also downstream processing.

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