Redox-Switchable Biocatalyst for Controllable Oxidation or Reduction of 5-Hydroxymethylfurfural into High-Value Derivatives

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ABSTRACT: Biocatalytic upgrading of biomass-derived 5-hydroxymethylfurfural (HMF) into high-value derivatives is of great significance in green chemistry. In this study, we disclosed the successful utilization of whole-cell Paraburkholderia azotifigens F18 for its switchable catalytic performance in the on-demand catalysis of HMF to different value-added derivatives, namely, selective reduction to 2,5-bis(hydroxymethyl)furan (BHMF) or oxidation to 5-hydroxymethyl-2-furancarboxylic acid (HMFXCA). Based on the fine-tuning of biochemical properties, the biocatalyst can proceed an efficient hydrogenation reaction toward HMF with a good selectivity of 97.6% to yield the BHMF at 92.2%. Noteworthily, BHMF could be further oxidized to HMFXCA and 2,5-furandicarboxylic acid (FDCA) by the whole cell. To realize the on-demand syntheses of HMFXCA, the genes encoding HMF oxidoreductase/oxidase of whole-cell F18 were then deleted to prevent the further conversion of HMFXCA to FDCA, which led to a 10-fold decrease of FDCA. Thus, an HMF conversion of 100% with an HMFXCA yield of 98.3% was finally achieved by the engineered whole cell at a substrate concentration of 150 mM. Moreover, HMFXCA synthesis was efficiently prepared with an excellent selectivity of 96.3% and a yield of 85.1% even at a high substrate concentration of up to 200 mM.

INTRODUCTION

The growing interest in the synthesis of nonoil chemical compounds has led to the development of renewable carbohydrate transformation into valuable chemicals. Recently, utilization of carbon-neutral biomass has attracted more interest in the production of bio-based platform chemicals. This trend is highlighted by the production of 5-hydroxymethylfurfural (HMF) derivatives as useful reagents. As a disubstituted furan, HMF can be produced by the dehydration of biomass-derived carbohydrates and has been listed as one of the “Top 10 + 4” value-added bio-based compounds. Owing to the presence of primary hydroxyl and formyl in the furan, it shows strong reactivity and could be transformed into valuable disubstituted furan derivatives via reduction, oxidation, etherification, and amination.

It is well known that both 2,5-bis(hydroxymethyl)furan (BHMF) and 5-hydroxymethyl-2-furancarboxylic acid (HMFXCA) are high-value building blocks, which have application potential in advanced materials, clean alternative fuels, and drugs. Currently, selective transformation of HMF to both derivatives is generally performed via a metal-catalyzed reaction at high temperatures/pressures. As a hydrogenation product of HMF, BHMF is commonly used for the synthesis of macrocycle polyether compounds and crown ethers. Traditionally, it is synthesized by the stoichiometric hydrogenation of HMF in the presence of lithium aluminum hydride (LiAlH4) or sodium borohydride (NaBH4). Recently, various chemocatalytic pathways, including traditional hydrogenation, transfer hydrogenation, electrocatalytic hydrogenation, and photocatalytic hydrogenation have been widely utilized in a comprehensive manner. Nevertheless, high pressures, organic solvents, and/or noble metals are still ineluctable during those reactions, thereby increasing the industrial and economic costs. Otherwise, the same problems exist in the synthesis of the HMFXCA, which is a partial oxidation product of HMF. Moreover, traditional chemical protocols also experience difficulties in generating this partly oxidized derivative in a selective fashion.

Biocatalytic reactions offer many benefits in the context of green chemistry because of the mild reaction conditions and being environmentally benign. In our previous works, different chemical methods for the transformation of HMF have been developed via the design of novel chemical catalysts. To realize the green preparation of valuable furan derivatives, biocatalysts with good selectivity and environmental friendliness were further explored as a promising alternative in our group. In fact, many inventions of the biocatalytic process have
been proposed to switch the transformation of HMF from chemical to biological approaches.\textsuperscript{6,9,11} Daniels’ group first found that HMF could be reduced by different types of bacteria into its alcohol as early as 1993.\textsuperscript{12} However, further studies cannot be performed because of the limitation of their analysis conditions at that time. In 2004, Liu et al. first isolated and purified the reduction product of HMF from the yeast-based conversion mixtures and identified it as BHMF.\textsuperscript{13} Subsequently, \textit{Meyerozyma guilliermondii} SC1103 whole-cell was proved to be active for the reduction of HMF, and more than 86% BHMF yield could be achieved using 100 mM HMF.\textsuperscript{6}

Apart from the reduction process, the biocatalytic oxidation of HMF also attracts huge interest. In recent years, many significant breakthroughs have been achieved in the complete oxidation of HMF for 2,5-furandicarboxylic acid (FDCA).\textsuperscript{14} However, few reports consider the partial oxidation of HMF to HMFCa because of the oxidized yet incomplete state of the product. Initially, enzyme-based approaches were reported for the selective production of HMFCa. An organocatalytic-enzymatic method was developed based on 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) and lipase CALB as catalysts, and 52% yield of HMFCa was obtained in eBuOH.\textsuperscript{15} To circumvent the usage of TEMPO, new specified enzymes were further explored for the oxidation of HMF. When aryl-alcohol oxidase from \textit{Pleurotus eryngii} was employed as a catalyst by Carro et al., the yield of HMFCa was up to 98%.\textsuperscript{16} Recently, another promising approach for the production of HMFCa has been put forward based on the whole cell. Nevertheless, most reported whole cells resulted in the vast coexistence of HMFCa and FDCA.\textsuperscript{17} One of the typical exceptions is \textit{Comamonas testosteroni} SC1588 which afforded a yield of approximately 98% HMFCa at a substrate concentration of 160 mM.\textsuperscript{18} However, the pathogenicity of the biocatalyst seriously limited its potential application. To explore green and economic routes for the upgrading of HMF, whole cells should be more preferable than the isolated enzymes in applicability. Compared to the preparative usage of cell-free isolated enzymes which are specific catalysts for a certain biotransformation, whole-cell biocatalysts offer a number of advantages such as easy handling, no need for cell lysis and enzyme purification, increased stability, and the presence of natural cofactors. Moreover, endogenous dehydrogenases could act synergistically in performing the transformation of HMF, thus enhancing the overall efficiency of the biocatalytic process.\textsuperscript{18}

Biocatalysis utilizing whole cells has come a long way from processes using wild-type microorganisms to tailor-made recombinant strains.\textsuperscript{15} Nowadays, most reported biocatalytic transformation of HMF focused on either the reduction to relative alcohol (BHMF) alone or oxidation to its carboxylic acids (HMFCa and FDCA).\textsuperscript{1,6,9} Otherwise, some whole cells resulted in the vast coexistence of different products at the same time, which have less practical value in industrial production.\textsuperscript{17} In the present work, a novel bacterium was explored from chemically polluted microhabitats by HMF enrichment and identified as \textit{Paraburkholderia azotifigens}. The HMF tolerance of the strain enabled it with high HMF metabolic ability even at high substrate concentrations. Key factors on the targeted conversion of HMF into different derivatives by the whole cell were systematically studied. To block the overoxidation process during the transformation, the unwanted genes encoding key enzymes of the whole cell were deleted via gene engineering. Finally, the combinatorial fine-tuning of biochemical characteristics and the synthetic pathway of the whole cell provides a novel method for the controllable oxidation or reduction of HMF into different value derivatives effectively.

## RESULTS AND DISCUSSION

### Screening and Characterization of HMF-Degrading Biocatalysts.

The high HMF tolerance of microbial cells is a prerequisite for the effective transformation of HMF because of the well-known biotoxicity of furan compounds.\textsuperscript{23} Initial experiments demonstrated that six strains of microbes isolated were able to grow well in the presence of 80 mM HMF. In the absence of glucose and peptone, no growth was observed, indicating that HMF could not be used as the sole source of carbon and energy for their growth. The growth of selected strains and their percentage transformation of HMF are presented in Table S1. After 12 h of incubation, all but strain K-16 transformed more than 51% of the HMF. Amazingly, the growing strain F-18 transformed approximately 98% of HMF, as monitored by the decrease in the absorbance at 282 nm. Plots of percent maximal growth versus the HMF concentration were constructed. The concentration of HMF that led to 50% inhibition of the growth of microorganisms (IC\textsubscript{50}) was estimated. Most of the strains had an IC\textsubscript{50} of above 26 mM, and the strain F-18 had an IC\textsubscript{50} value of 59.2 mM. At the same concentration of HMF, HMF-degrading bacterial strains \textit{Citrobacter freundii} and \textit{Edwardiella} sp. afforded the IC\textsubscript{50} values of 21 mM and 26 mM, respectively, as reported earlier.\textsuperscript{24} The exponential growth phase of strain F-18 in HMF was observed for 48 h and growth was found to be luxuriant in the presence of HMF less than 200 mM, while no growth was observed in medium with the concentration up to 300 mM (data not shown). These results indicated the excellent HMF tolerance of strain F-18. The isolated strain F-18 was characterized further, and its 16S rRNA sequence showed 98.1% identity and 80% query cover with \textit{Paraburkholderia} species, as shown in the Supporting Information. The isolate was then named \textit{P. azotifigens} F18.

### Analysis of the Conversion Curve of HMF Catalyzed by Whole-Cell F18.

The native transformation ability of the biocatalyst \textit{P. azotifigens} F18 toward HMF was studied. Figure 1 shows the pattern of HMF conversion by the whole-cell biocatalyst. Initially, the concentration of HMF decreased quickly, and BHMF was synthesized in large amounts. More than 30 mM BHMF was obtained within 6 h, and most of the HMF (85.7%) was converted to HMF alcohol, with only 4.1 mM HMFCa obtained. With the extension of time, the amount of BHMF diminished quickly, and then more HMFCa was detected. HMF completely transformed within 18 h. It is noteworthy that massive accumulation of HMFCa was observed after 24 h, and the maximum amount of HMFCa (31.2 mM) was found at 42 h with a small amount of FDCA (8.7 mM) detected. The continuous increase of FDCA after 12 h indicated the existence of HMF oxidoreductase (HmfH) or HMF oxidase (HmfO) in the whole cell, which could oxidize HMF alcohol and HMF acid to form FDCA.

After prolonged bioconversion of HMF by whole-cell F18, nearly 24 mM HMFCa and 15 mM FDCA were finally obtained, as shown in Figure 1. No other products were detected anymore, indicating the relatively simple enzyme systems in whole-cell F18. These results suggest that HMF might be first converted to HMF alcohol, then re-oxidized to HMF at a harmless lower concentration, and subsequently...
oxidized to its acid forms. The HMF biodegradation pattern by P. azotigenes F18 was similar to that of Raoultella ornithinolytica BF60 as reported before but with less accumulation of FDCA. Similar phenomena have also been confirmed in the common biodetoxification mechanism of microorganisms toward furan. Overall, HMF can be converted not only to its corresponding alcohol but also to its corresponding acid by P. azotigenes F18 whole cell. Further adjustment of the intracellular enzyme activity might regulate the targeted conversion of HMF into the expected valuable products.

Regulation of the Catalytic Activity of Whole-Cell F18 for the Targeted Synthesis of BHMF. Age-Related Regulation of the Whole Cell on BHMF Synthesis. The gene expression level of microbes varies in different growth phases, thus the core enzymes of the HMF metabolic pathways might be affected by the age of the whole-cell. The catalytic performances of whole-cell F18 with different ages (growth for 4 to 24 h) were first studied in the transformation of HMF into BHMF. As analyzed above, three types of products, including BHMF, HMFCA, and FDCA, could be formed with the extension of the reaction time, but the type of product has strong correlation in time. The optimal reaction time for the BHMF production was observed at 12 h. Figure 2a shows the age-related influence on HMF transformation. In general, the cell under the logarithmic phase displayed a much higher reducibility in the production of BHMF. As the cultivation age increased, the selectivity of the whole-cell was significantly decreased. As is shown, the yield of BHMF was approximately 64.5% (25.8 mM) with the catalysis of the 4 h cell toward 40 mM HMF, and the selectivity achieved was as high as 100%. However, when the 8 h cell was adopted, the yield of BHMF increased to 72.8% (29.1 mM), but the selectivity decreased to only 89.2%. HMF was completely transformed by the 12 h cell within 12 h, but the catalytic specificity of whole-cell F18 decreased further and a small amount of FDCA (1.9 mM) was also detected. Meanwhile, under the catalysis of the 24 h-cell, more HMF was oxidized to HMFCA with the yield of 39.8% (15.9 mM), along with the formation of more amounts of the overoxidized product FDCA (7.6 mM). It was presumed that the genes for aldehyde oxidase might be expressed quickly and earlier than those for aldehyde reductase. This could be supported by the fact that the HMF degradation pathway of microbes started with the quick degradation of HMF into its less toxic metabolite HMF alcohol and then into HMF acid.

To further detect the catalytic performances of the 4 h cell, the effect of reaction time on the yield and selectivity of BHMF was analyzed. As described in Figure 2b, excellent selectivity (100%) was obtained only within 12 h, and the yield achieved was 66.3% (26.5 mM). When the reaction time reached 18 h, the selectivity of BHMF decreased significantly to 69.4%, although the transformation rate of HMF increased to 79.5%. It was deduced that BHMF might be degraded further by the subsequent expressed oxidases inside the whole cell with the extension of reaction time. A significant increase of the cell mass indicated the exuberant metabolism of cells in the presence of glucose. Finally, although HMF was completely transformed, the poor selectivity of BHMF (30.2%) was obtained after 30 h. Moreover, both HMFCA and FDCA were detected in the reaction with the yield of 48.5% (19.4 mM) and 14.7% (5.8 mM), respectively. Those highlighted the
crucial roles of cell age and reaction time in the whole cell-catalyzed synthesis of BHMF and indicated that the level of age-related enzymes might significantly change the catalytic performance of whole-cell F18.

**Effect of Aerobic and Anaerobic Conditions on BHMF Synthesis.** Oxygen levels significantly affect the activity of intracellular oxygen-dependent alcohol oxidases/aldehyde oxidases, which could be used to regulate the whole-cell-catalyzed conversion of HMF.26 The catalytic performance of *P. azotifigens* F18 (4 h-cell) toward HMF was then tested under aerobic and anaerobic conditions. The control experiment under aerobic conditions without microbes indicated that the aeration did not lead to the direct oxidation of HMF/HMF alcohol at ambient temperature (data not shown). Figure 3 shows the impact of the aerobic conditions on BHMF synthesis with different oxygen contents. Reaction conditions: whole cell (2 g, wet weight, 4 h growth), phosphate buffer (30 mL, 100 mM, pH 7.0) containing HMF (40 mM), and glucose (20 mM) were incubated at 30 °C and 150 rpm. The control reaction was performed in an anaerobic tube made by evacuation of air and using N2 to replace the air.

The BHMF yield of 70.3% (28.1 mM) was obtained with 20 mM glucose, while being up to 92.2% (36.9 mM) with 40 mM glucose. Moreover, the selectivity of BHMF increased from 93.1 to 97.6%. Glucose is the preferred cosubstrate in this reaction, possibly due to more production of the nicotinamide cofactor (NAD(P)H) in cells. As a reducing agent, NAD(P)H is important for the reduction of HMF into BHMF.25 The results might indicate that reversible reactions exist in this synthetic reaction.

In the case of glycerol, lower yield (50.2%) and selectivity (82.7%) were achieved with more formation of HMFCA. In addition, more HMFA and FDCA were produced under aerobic conditions.

**Targeted Synthesis of HMFA by Combinatorial Gene Deletion in Whole-Cell F18.** Although high yield and selectivity of BHMF were achieved with the catalysis of whole-cell F18 as shown above, vast accumulation of HMFA was also noted with the prolongation of reaction time. Therefore, construction of the effective synthesis pathway of HMFA based on genetic engineering in *P. azotifigens* F18 was also noted with the prolongation of reaction time.

The alcohol oxidases and/or aldehyde oxidases with free oxygen as a substrate for the oxidation of HMF alcohols were effectively restrained under anaerobic conditions.25 These results also verified that HMF was first converted to HMF alcohol without the presence of oxygen in this biocatalyst and then oxidized to its acid forms.

**Effect of Cosubstrates on BHMF Synthesis.** Cosubstrates exerted a significant effect on the catalytic performance of the whole cell for regulating the reducibility of the biocatalyst, as well as for fixing the damage caused by toxic furans.27 In this study, the BHMF yields were closely dependent on the glucose concentrations under anaerobic conditions. In general, the catalytic performance of the whole cell was improved with higher glucose concentrations, as shown in Figure 4. The
would be significant. Recently, the HMF degradation pathway of *Cupriavidus basilensis* HMF14 has been characterized. First, HMF was converted to HMF acid either by oxidoreductase HmH or by specific aldehyde dehydrogenases. Second, HMF acid was further oxidized to FDCA, which was catalyzed solely by HmH, making this enzyme a promising key biocatalyst for the production of FDCA. It was speculated that preventing the formation of FDCA by deletion of the genes responsible for the oxidation of HMFO in *P. azotigen* F18 could improve the targeted synthesis of HMFO.

*Deletion of the Genes hmfH and hmfO for HMFO Production.* To investigate the positive mutant of whole-cell F18 for HMFO production, genes that are responsible for HMF oxidoreductase (*hmfH*) and HMFO oxidase (*hmfO*) in this strain were deleted to block FDCA production, and then whole-cell bioconversion experiments were carried out using the engineered cell and 150 mM HMF as the substrate. The *λ*-red recombination system was adopted to create an insertional mutant of *hmfH*, the gene encoding HMF oxidoreductase for the oxidation of the corresponding monocarboxylic acid and thereby reducing HMFO degradation. When the gene of HMF oxidoreductase (*hmfH*) was deleted in *P. azotigen* F18, no significant improvement of yield was found as expected. Only a slight increase of the HMFO yield (from 103.5 to 113.4 mM) was obtained compared to the wild F18 strain at 42 h (Figures S1 and S2). It has been reported that the *C. basilensis* HMF14 mutant with a disrupted *hmfH* gene accumulated HMF acid when cultured in the presence of HMF. Cell extracts of wild-type *C. basilensis* HMF14 expressing HmH formed FDCA when incubated with HMF acid, confirming that HmH catalyzes the oxidation of HMFO to its dicarboxylic acid form. The limited improvement in our mutant strain indicated the incomplete disturbance of the following metabolic pathway of HMFO. The reasons might be that HMFO can also be oxidized by HMF oxidase (HMFO) via route B or other endogenous nonspecific dehydrogenases in route A (Scheme 1). Therefore, the HMFO gene that is responsible for the oxidation of HMFO was further deleted to verify its functions. The gene manipulation process and the verification experiment are shown in the Supporting Information. As a result, the yield of HMFO was increased from 103.5 to 133.4 mM after 42 h of reaction, as shown in Figure S3. This indicated that both *hmfO* and *hmfH* were responsible for HMFO oxidation to form FDCA in strain F18. Based on this finding, the final engineered strain was designed harboring two gene deletions: *hmfH* and *hmfO*. The combination gene deletion was performed to investigate the synergistic function of both genes. Excitingly, a significant increase in the production of HMFO was observed after deleting *hmfO* and *hmfH* together. The time course of HMFO production from HMF showed that the biotransformation yield achieved was 100% from 42 h, along with the production of 94.1% HMFO (141.2 mM), 4.4% BHMF (6.6 mM), and 2.5 mM FDCA (Figure 5). Moreover, deletion of target genes did not affect the cell growth. Finally, the HMFO yield achieved was 98.3% (147.5 mM) at 48 h, with only 2.5 mM FDCA detected. Similarly, Li et al. have reported the whole-cell biocatalytic selective oxidation of HMF to HMFO. Excellent substrate conversions (approximately 100%) and good HMFO yields (88–99%) were obtained when the substrate concentrations were less than 130 mM. However, the biocatalyst did not show any reduction activity and was highly sensitive to the acidity of the product.

*Improved Synthesis of HMFO with the Tuning of Acidity and Dissolved Oxygen.* Based on the excellent HMF tolerance of *P. azotigen* F18, a reaction was carried out on a preparative scale at the limit-substrate concentration for cell growth (200 mM). As is known, vast accumulation of acid products in a reaction would significantly restrain the activity of the biocatalyst. Thus, the pH of the reaction mixture was tuned to eliminate the detrimental effect of the oxidized acid products. In the natural process of the synthesis of HMFO, the pH decreased from 7.0 to 4.2 with the prolongation of the reaction time. Continuous pH tuning for a neutral environment (7.0) proved to be effective for improving the yield of the desired product from 71.3 to 81.5%. In addition, four oxygen vectors including *n*-hexane, *n*-dodecane, *n*-hexadecane, and oleic acid were added into the reaction medium for HMFO production (Figure S4). Among these four oxygen vectors, oleic acid and *n*-hexane showed a negative impact on the HMFO production, whereas *n*-dodecane and *n*-hexadecane

**Scheme 1. Putative Pathways for the Oxidation and Reduction of HMF in Bacteria; Polygons Indicate Enzymes with the Following Activities: Triangle, HMF Oxidoreductase (*HmH*); Tetragon, HMF Oxidase (HMFO); Circle, Probably Nonspecific Dehydrogenases—DFF, 2,5-diformylfuran; FDCA, 2,5-Furandicarboxylic Acid; HMF, 5-Hydroxymethylfurfural; HMFC, 5-Hydroxymethyl-2-furoic Acid; BHMF, 2,5-Bis(hydroxymethyl)furan**

**Figure 5.** Time course of HMFO production based on the catalysis of engineered *P. azotigen* F18 with combinatorial gene deletion. Wet cells (4.0 g) were resuspended in 30 mL reaction buffer (pH 7.0) containing HMF (150 mM). The cosubstrate glucose (20 mM) was added. The reactions were carried out at 30 °C and 200 rpm.
increased the activity of the biocatalyst to different extents. Among these two oxygen vectors, n-hexadecane exhibited the most enhancement on the HMFCA production. After the addition of 1% (v/v) n-hexadecane, an excellent selectivity of 96.3% and a yield of 85.1% were achieved. Moreover, the reaction periods decreased significantly from 60 h to 36 h and excellent selectivity was retained during the reaction. After the reaction in this work, the isolation of HMFCA was further conducted. The pH of the reaction mixture was tuned to 1.0, followed by 3 times extraction with ethyl acetate. The removal of the solvent via evaporation afforded the product with an isolated yield of 99% and a purity of 98%. In fact, although high conversion efficiency and selectivity were achieved in previous reports, the biocatalytic transformation of HMF still faces the unavoidable issues of substrate bio-toxicity. Initially, the HMF concentration during the biocatalysis process was almost less than 20 mM.1,16 Recently, with the exploration of the HMF tolerance biocatalyst, the transformation can be performed at concentrations up to 200 mM.11 To fulfill the industrial scale-up synthesis of HMFCA, it is the direction of long-term efforts to explore the HMF tolerance mechanism of biocatalysts.

■ CONCLUSIONS

Eco-friendly reactions for the efficient synthesis of BHMF or HMFCA were developed based on a novel whole-cell biocatalyst. The whole cell showed two different states as a highly active catalyst for the oxidation and reduction of HMF, and both reactions could be turned “off” and “on” by reaction engineering. Based on the tuning of the biological activity of the biocatalyst, BHMF was first successfully synthesized from HMF. Most BHMF was further transformed into HMFCA with the prolongation of reaction time. Thus, the enzymes responsible for the oxidation of HMF in the whole cell were knocked to block the formation of FDCA, and then HMFCA synthesis was significantly improved. Finally, both desired products could be readily obtained with good yields and excellent selectivity. The biological approaches employed in this study were green. Moreover, for the first time, an enlightening method for the on-demand conversion of HMF into high-value derivatives was provided based on the fine-tuning of one type of biocatalyst.

■ EXPERIMENTAL SECTION

Chemical Reagents. HMF (99%) and other standard substances, including HMFA (98%), BHMF (98%), and FDCA (98%), were purchased from Energy Chemical Scientific Ltd. (Shanghai, P. R. China). Other chemicals were of analytical grade unless otherwise specified and purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). DNA purification kits, spin column plasmid miniprep kits, and T4 DNA ligases were purchased from Takara (Dalian, China). The primer synthesis was done by Nanjing GenScript Biotech Co., Ltd. (Nanjing, China).

High-Throughput Screening of the Biocatalyst with Excellent HMF Tolerance. The microbes were isolated from soil samples of a crude oil field in Shandong Province of China. HMF was added to the screening medium at selective pressures. A high-throughput screening method was developed based on the detection of cell growth in the presence of 80 mM HMF and residual HMF remaining in the medium. Microbes were isolated using the traditional enrichment technique. Then, clones were transferred into glucose media added with 80 mM HMF in a 96-well plate. Plates were incubated for 24 h at 30 °C and shaken at 200 rpm. The culture suspensions were detected at OD600. Furthermore, residual HMF in the culture supernatant was measured by colorimetry. Briefly, the supernatant was diluted with water to obtain a reaction solution after the addition of Carrer solution I and Carrer solution II.20 The mixture was filtered and placed in tubes. Deionized water and 0.2% sodium bisulphide solution were added to different tubes, respectively. The absorbance of the reaction solution at 284 and 336 nm was determined versus that of the reference solution.21 Clones showing lower levels of residual HMF recovery and a higher growth rate in HMF were selected for the further detection of related derivatives. Finally, a F18 strain identified as P. azotifigens was chosen based on its excellent characteristics.

Procedure for the Biocatalytic Reduction of HMF to BHMF. Typically, a phosphate buffer (30 mL, 100 mM, pH 7.0) containing HMF (40 mM), glucose (20 mM), and the microbial whole cell (2 g, wet weight, 4 h) was incubated at 30 °C and 150 rpm. The reaction was then performed in an anaerobic tube made by evacuating air with a water vacuum and using N2 to replace the air. At specified time points, aliquots were withdrawn from the reaction mixtures and analyzed by high-performance liquid chromatography (HPLC). The yield was defined as the ratio of the measured product amount to the theoretical product amount based on the initial amount of HMF. The selectivity was defined as the ratio of BHMF amount to the sum of all the products. All experiments were conducted in triplicate.

Construction of Gene Knockout Mutants of the Biocatalyst P. azotifigens F18. To create a mutant of P. azotifigens F18 for the targeted synthesis of HMFA, two key genes restricting the synthesis process were knocked out. The genes encoding HmFO (ADQ83320.1) from Methylavorus sp strain MP688 and HmHF (ADE20408.1) from C. basileiensis HMF14 were used as templates for the design of primers to clone hmfH and hmfO genes in P. azotifigens F18. The gene encoding HMF oxidoreductase (hmfH) was first mutated in order to prevent the conversion of HMFA to FDCA. The gene encoding HMF oxidase (hmfO) was further mutated to prevent the conversion of HMF via route B (Scheme 1).22 The native sequences of the selected genes cloned from P. azotifigens F18 are shown in the Supporting Information. The gene deletion was performed using the λ-Red recombination system. The primers used for plasmid construction and gene deletion are shown in the Supporting Information (Tables S2 and S3 and Figure S5).

General Procedure for the Biocatalytic Oxidation of HMF to HMFA. The experiment for the oxidation of HMF to HMFA with engineered P. azotifigens F18 whole cells (growth for 12 h) was performed in a 50 mL Erlenmeyer flask. Wet cells (4.0 g) were resuspended in 30 mL of a reaction buffer (pH 7.0) containing HMF (40–200 mM). Cosubstrates such as glucose (20–60 mM) were added. The reactions were carried out at 30 °C and 200 rpm. Quantitative samples were taken and extracted with ethyl ether, and the organic phase was volatilized and dissolved by ethanol and then subjected to HPLC analysis for the determination of the yields. All experiments were conducted in triplicate.

Preparation of the Whole-Cell Biocatalyst. For the preparation of seed cultures, the P. azotifigens F18 strain was incubated for 12 h at 35 °C in Gauze’s Synthetic Medium no. 1
without or with gentamicin (50 mg·L⁻¹) for the wild-type strain and mutant strain, respectively. The medium used for the propagation of P. azotifigens F18 was a minimal medium (MM), as described previously. Glucose and tryptone were added to the MM. Antibiotics were added as required with or without gentamicin in a mineral medium. All shake-flask cultures were performed in Erlenmeyer flasks stoppered with paper plugs. The seed cultures (2%, v/v) were then inoculated into the MM. After incubation at 35 °C for a definite time, the cells were harvested by centrifugation, and the whole-cell was then resuspended in PBS and kept at 4 °C for further use.

Analysis Methods. The reaction mixtures were determined by HPLC (1260 series; Agilent, Santa Clara, CA, USA) on a Spurasil C18-EP column (4.6/250 mm, 5 μm, Agilent, USA) at 30 °C, with elution performed using acetonitrile and phosphate-buffered saline (PBS) solution (80:20, v/v) with a flow rate of 1.0 mL·min⁻¹. The detection was performed with a UV detector at a fixed wavelength of 230 nm. The HPLC retention time values of the standards of HMF, BHMF, and FDCA were 6.44, 5.55, 4.73 and 4.0 min, respectively (Figure S6). The yield was defined as the ratio of the measured product amount to the theoretical product amount based on the initial amount of HMF. The selectivity was defined as the ratio of certain product amount to the sum of all the products. All experiments were conducted in triplicate.
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