

Article

Efficient Production of 5-Hydroxymethyl-2-Furfurylamine from Expired Beverage Waste via Chemo-Biological Cascade Reaction for Bridging Chemocatalysis and Biocatalysis

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Abstract

In this study, expired beverage (EB) waste was converted into 5-hydroxymethyl-2-furfurylamine (HMFA) through a chemoenzymatic cascade in a malonic acid–choline chloride (MA:ChCl) deep eutectic solvent (DES) system. Under the optimized conditions, HMF was produced from expired beverage waste at 160 °C for 60 min using an MA:ChCl-to-EB mass ratio of 1:5. A recombinant *Escherichia coli* strain co-expressing pyruvate decarboxylase and the engineered ω -transaminase LF was constructed and used to convert EB-derived 5-hydroxymethylfurfural (HMF) into HMFA with a yield of 96.6%. This biological amination of HMF can be conducted under mild conditions via amination (37 °C, 12 h), while reducing the required *D*-alanine loading (from *D*-alanine/HMF = 16/1 to *D*-alanine/HMF = 4/1, based on molar ratio). This two-step chemoenzymatic cascade provides a promising route for producing high-value biofuran compounds from food waste.

Keywords: 5-hydroxymethyl-2-furfurylamine; HMF; chemobiocatalytic approach; ω -transaminase; deep eutectic solvent

1. Introduction

The depletion of non-renewable resources, particularly fossil fuels, and growing environmental concerns have increased interest in renewable carbon resources [1–3]. Accordingly, the utilization of abundant, inexpensive, and renewable biological resources has emerged as a key focus for manufacturing high-value biobased compounds, biopolymers, and biomaterials [4–9]. To realize the valorization of food waste, recycling strategies including composting, anaerobic digestion, and repurposing as animal feed can significantly reduce greenhouse gas emissions and support a circular bioeconomy [10–13]. Various food wastes (e.g., bread waste, fruit waste, vegetable waste, and beverage waste) are typically rich in organic matter, which can generate environmental pollutants, such as leachate and odors, if not appropriately treated [14,15]. Consequently, it is essential to adopt proper processes to recover resources from organic waste, such as expired dairy products, to promote circularity and support the realization of a green economy [16].

Various bioresources are extensively adopted as feedstocks for producing 5-hydroxymethylfurfural (HMF) via homogeneous and heterogeneous chemocatalysts [17–22],



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including Cr-zeolites, HSO_3 -ZSM-5 zeolite, Sn-beta zeolite nanocrystals, UiO-66 metal-organic framework, sulfosuccinic acid-based metal-center, tin-doped sulfonated activated carbon, $\text{Ti-Al}_2\text{O}_3$, Sn Amberlyst-15 resin, AlCl_3/HCl , etc. [23–26]. Recently, there has been growing interest in the manufacturing of HMF using deep eutectic solvents (DESs), which can serve as chemocatalysts, additives, and reaction media [27]. DESs are generally formed by mixing a hydrogen-bond donor and a hydrogen-bond acceptor at an appropriate molar ratio, resulting in extensive hydrogen-bonding interactions [28–30]. They have attracted considerable attention because of their low cost, ease of preparation, tunable properties, and potential biodegradability [31,32]. Organic acid-based DESs can serve as both acidic catalysts and reaction media for the dehydration of C6 sugars into HMF.

5-Hydroxymethyl-2-furfurylamine (HMFA) is one main derivative of HMF [33], where the aldehyde group ($\text{C}=\text{O}$) is substituted by an amine group ($-\text{NH}_2$). It is commonly utilized in the production of curing agents, polymers, food additives, drugs, and agricultural chemicals. HMFA can be biosynthesized through the ω -transaminase-catalyzed amination of HMF. In the presence of pyridoxal 5'-phosphate as a coenzyme, the amino group is transferred from an amine donor to the substrate, which acts as the amino-group acceptor with ω -transaminase under mild conditions [34,35]. However, the use of large amounts of costly amine donors and long reaction times poses major challenges that may hinder the industrial application of ω -transaminases [36]. A promising strategy for improving bioamination is the use of multienzyme cascade reactions. These engineered cascade bioreactions are adopted to drive unfavorable equilibria. It is therefore important to develop a multienzyme cascade that reduces amine-donor consumption, improves catalytic efficiency, and enables cost-effective HMFA production. ω -Transaminase derived from *Aspergillus terreus* is an effective enzyme for the production of organic amines. One of the common methods to enhance the formation of organic amines is utilizing *D*-alanine (*D*-Ala) as an amine donor and eliminating pyruvate or acetone by-products. Pyruvate can be eliminated by decarboxylation reactions through the biotransformation with pyruvate decarboxylases, forming acetaldehyde (CH_3CHO) and carbon dioxide (CO_2). Consequently, removal of pyruvate shifts the reaction equilibrium toward continuous amine formation (e.g., HMFA). This can considerably accelerate the biological amination reaction and diminish the excessive load of *D*-Ala (Figure 1).

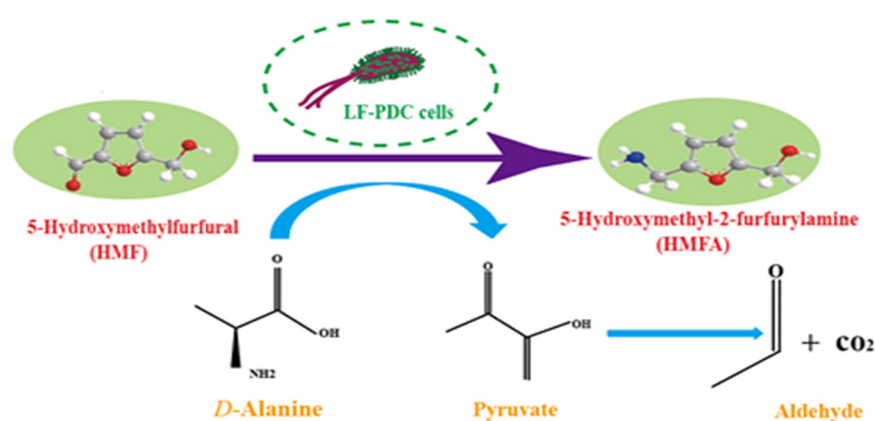


Figure 1. Co-expression of ω -aminotransferase (LF) and pyruvate decarboxylase (PDC).

Although ω -transaminases are attractive biocatalysts for the synthesis of amino-functionalized compounds, naturally occurring enzymes often exhibit limited activity toward non-natural aromatic aldehyde substrates such as HMF. Therefore, protein engineering strategies have been employed to improve substrate recognition and catalytic efficiency. In particular, an engineered ω -transaminase derived from *Aspergillus terreus*

containing an L187F mutation (LF mutant) was reported by our group to exhibit enhanced HMF transamination activity, enabling efficient synthesis of HMFA. Accordingly, the LF mutant was selected in this study as the aminotransferase component of the whole-cell cascade. Lactic acid bacteria-based beverages contain high sugar content, and expired or discarded beverages can be used as feedstocks for the production of biobased chemicals. To transform food waste-derived HMF into HMFA via transaminase, developing an efficient bioprocess is therefore of considerable interest. In this research, DES MA:ChCl (MA-to-ChCl 2:1, molar ratio) was prepared, and recombinant *E. coli* cells harboring PDC and transaminase LF [Leu (L) at AT187 was mutated to Phe (F) in *Aspergillus terreus* transaminase] were created. A tandem chemoenzymatic process was developed to produce HMFA from expired beverage (EB) waste through tandem catalysis with the DES MA:ChCl chemocatalyst and LF biocatalyst in MA:ChCl. The effects of chemocatalysis temperature, reaction duration, EB loading, and catalyst dose on the formation of HMF were evaluated. Afterward, the optimization of biocatalysis factors, including bioreaction temperature, medium pH, amine-donor dosage, and substrate loading, was implemented. In MA:ChCl, HMF was biologically aminated with a diminished amount of amine donor to yield the high-value product HMFA, thereby providing a potentially sustainable route for producing biobased chemicals.

2. Results and Discussion

2.1. Bioamination of HMF

Currently, *D*-Ala is recognized as a viable amine donor for (*R*)-type ω -transaminase, and an excess of *D*-Ala is commonly required to achieve optimal bioamination efficacy [37]. Conventional bioamination often requires a high amine-donor loading, resulting in inefficient use of raw materials. Conversely, PDC removes the pyruvate coproduct by converting it into acetaldehyde and CO₂, thereby shifting the transamination equilibrium toward HMFA formation. In some studies, transaminases can be co-expressed with auxiliary enzymes, resulting in diminished co-substrate loading [38,39]. In this research, ω -transaminase derived from *Aspergillus terreus* was selected as the principal biocatalyst for HMF amination in the process of biological amination. Additionally, PDC was connected in tandem to form a double enzyme expression vector for bioamination [40]. During LF-catalyzed transamination, *D*-Ala donates its amino group to HMF and is converted into pyruvate. Co-expressed PDC subsequently decarboxylates pyruvate to acetaldehyde and CO₂, thereby pulling the transamination equilibrium toward HMFA formation and reducing the required *D*-Ala loading. Reaction pH and temperature are important parameters for amination of HMF by recombinant whole-cell catalysts containing ω -transaminases. In addition, the effects of MA:ChCl DES concentration in the bioreaction system need to be evaluated because DES can enhance HMF solubilization and act as the reaction medium, whereas excessive DES concentrations may reduce cellular activity. Finally, different HMF concentrations need to be examined to evaluate substrate tolerance and the applicability of the whole-cell catalyst under high substrate loading.

Selecting an appropriate amine-donor loading is crucial to enhancing amination activity [41,42]. Figure 2A shows that amination activity increased markedly as the *D*-Ala/HMF molar ratio increased from 1:1 to 4:1 (mol/mol). Over 1:4 (mol/mol), the catalytic activity showed no further significant change, which was likely because *D*-Ala (amine donor) was already present in excess in catalyzing the bioamination of HMF [43]. Accordingly, the proper HMF/*D*-Ala molar ratio was 1:4. Supplementing DES might impact the biological amination activity, and biotransformation of HMF was implemented in the presence of the DES MA:ChCl (0–10 wt%) at pH 8.5 (adjusted with 1 M NaOH) by using 200 mM HMF as substrate. Notably, no substantial inhibition was observed at

MA:ChCl concentrations of 2–6 wt% (Figure 2B). Over 8 wt%, the bioamination activity dropped slightly. At 10 wt%, the inhibition effect was nearly 10%.

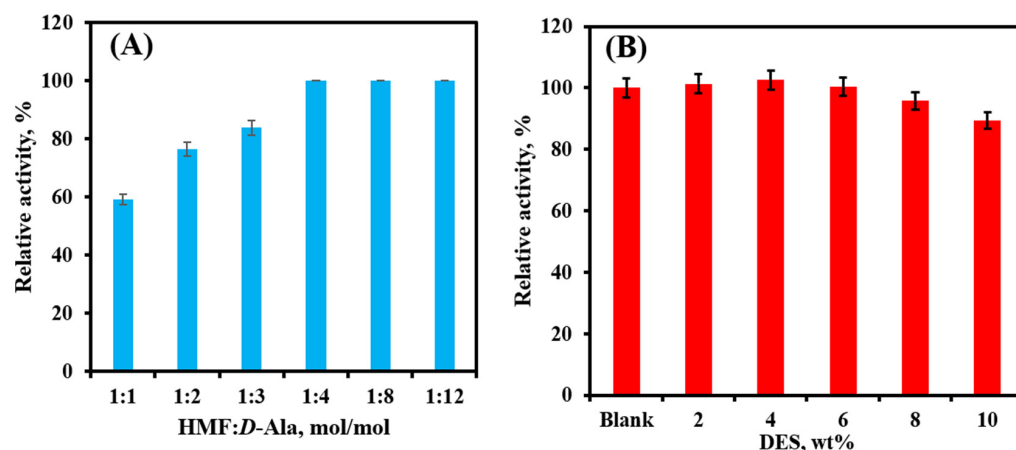


Figure 2. Effect of *D*-alanine addition on the biotransformation activity (A); effect of DES loading on the biotransformation activity (B). [The highest amination reaction rate value in each experiment was defined as 100% relative activity; all experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

The biological reaction conditions had an apparent impact on the biocatalytic activity [41,44,45]. The effects of bioreaction temperature (20–50 °C) and bioreaction medium pH (4–10) on manufacturing HMFA via biotransamination were estimated in the aqueous system containing *E. coli* LF-PDC whole cells. Upon undergoing amination at different temperatures, a crucial impact on the bioamination reaction might occur (Figure 3A). At 37 °C, the biocatalytic activity reached the highest level. As the amination temperature was higher than 37 °C, protein denaturation might occur [43], resulting in a decreased bioamination efficiency. The medium pH might impact the affinity of the enzyme and substrate [46,47]. It was observed that the pH of the medium (4.0–10.0) had a substantial impact on the transaminase activity, and the highest activity was observed at pH 8.0 (Figure 3B).

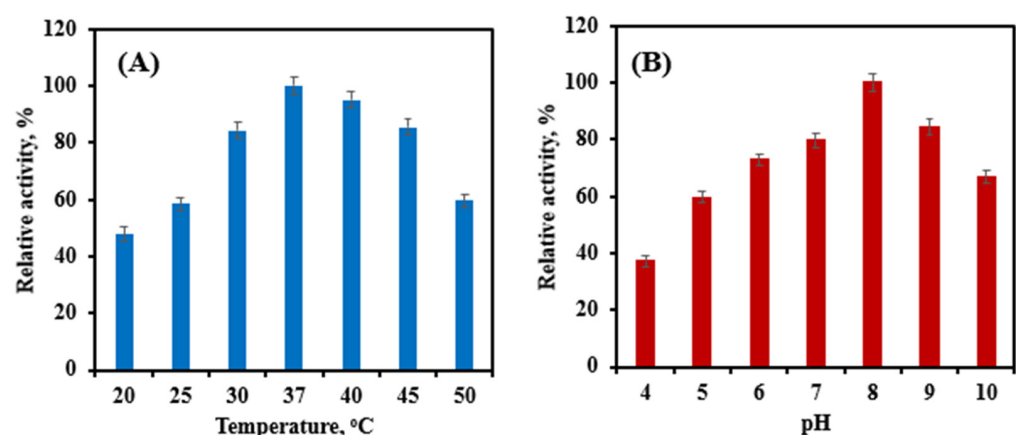


Figure 3. Effect of temperature (A) and pH (B) on the biotransformation activity of *E. coli* LF-PDC whole cells. [The highest amination reaction rate value in each experiment was defined as 100% relative activity; All experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

The optimal temperature and pH observed in this study represent the overall performance of the recombinant *E. coli* LF-PDC whole-cell biocatalytic system rather than the intrinsic catalytic optima of purified LF or PDC enzymes. In whole-cell catalysis, the apparent activity is determined by multiple factors, including enzyme catalytic efficiency, in-

tracellular enzyme stability, substrate transport across the cell membrane, cellular metabolic activity, and maintenance of cellular integrity. The highest HMFA production at 37 °C is consistent with the physiological growth temperature of *E. coli*, where cellular metabolism and intracellular catalytic processes are generally maintained at high levels. Although purified ω -transaminases may exhibit different optimal temperatures depending on their structural characteristics, the whole-cell system requires a compromise between enzyme activity and host-cell stability. At elevated temperatures, decreased HMFA formation may result from reduced cell viability, impaired membrane integrity, and partial inactivation of intracellular LF and PDC. Similarly, the optimal pH of 8.0 reflects the combined response of the recombinant cells and the expressed enzymes. The pH environment influences not only LF and PDC catalytic activity but also intracellular ion balance, substrate uptake, and enzyme stability within the host cells. Although purified LF may show a specific pH optimum in vitro, the optimal pH for whole-cell biotransformation can shift due to intracellular buffering capacity and transport limitations. Therefore, the temperature and pH values obtained should be considered as operational conditions optimized for the LF-PDC whole-cell catalytic system rather than the absolute enzymatic optima of isolated LF and PDC proteins.

2.2. HMF Tolerance of *E. coli* LF-PDC Whole Cells

The capacity of a biocatalyst to tolerate highly loaded substrates is an important parameter for evaluating biocatalytic performance [48]. The optimal biological reaction conditions were used to assess the capability of whole cells of *E. coli* LF-PDC to catalyze the production of HMFA from different HMF concentrations (300, 400, 500, and 600 mM). The biotransformation capacity of *E. coli* LF-PDC whole cells demonstrated an initial increase, followed by a decline at elevated HMF concentrations. When HMF loading was less than or equal to 500 mM, no substrate inhibition was observed, and HMFA was obtained in an excellent yield (>92.8%) within 24 h (Figure 4). Upon undergoing amination of HMF at 600 mM, the conversion efficiency decreased substantially, and the HMFA yield dropped to 51.3%.

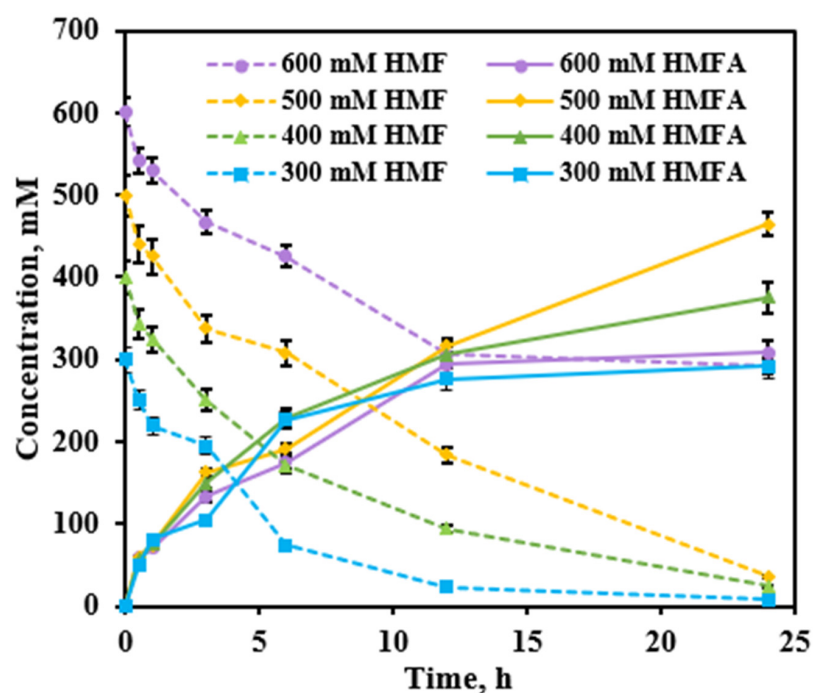


Figure 4. Time course of amination of different concentrations of HMF by *E. coli* LF-PDC whole-cell. [All experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

2.3. HMF Production from EB in MA:ChCl

It is recognized that DESs can serve as both chemocatalysts and reaction media for HMF production [49,50]. C6 sugars present in monosaccharides, disaccharides, polysaccharides, food wastes, and lignocellulosic hydrolysates are common feedstocks for HMF production [25,50–52]. In this research, acidic DES MA:ChCl was prepared and evaluated as an acidic catalytic medium to catalyze the generation of HMF from EB (33.3 g). Reaction time, reaction temperature, substrate load, and chemocatalyst dosage significantly affect the preparation of HMF [53–55]. To further improve the productivity of HMF, three main parameters were tested: dehydration temperature (150–180 °C), dehydration time (30–75 min), DES-to-EB mass ratio (0–8 wt%). HMF was prepared from EB at 150–180 °C for 30–75 min (Figure 5). Upon elevating reaction duration at 160 °C, the concentration of the formed HMF increased, and the maximum concentration was 261.2 mM in 60 min and then dropped to 240.8 mM in 75 min. In contrast, the yields of HMF reached the maximum at 60 min, 45 min, and 30 min at reaction temperatures of 150 °C, 170 °C, and 180 °C, which were 224.9 mM, 244.6 mM, and 215.7 mM, respectively. Further degradation of sugars and HMF into by-products, such as humins and levulinic acid, might occur at higher temperatures or prolonged reaction time [56,57], resulting in a reduced yield of HMF. Accordingly, the highest HMF concentration was obtained at 160 °C for 60 min.

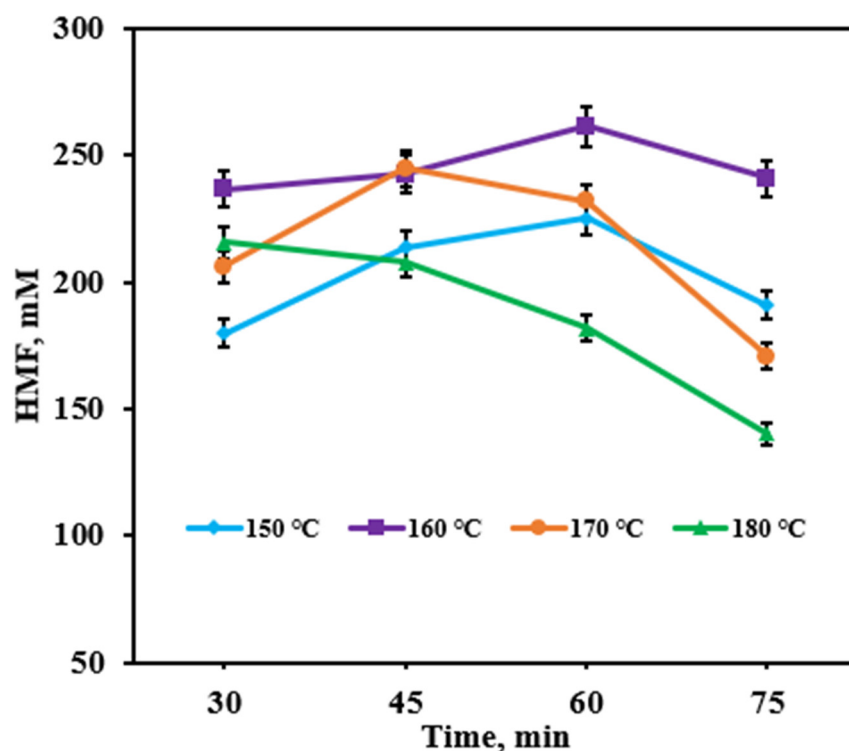


Figure 5. Effects of catalytic temperature and time on HMF production from expired beverages. [All experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

In the catalytic medium, the substrate-to-catalyst ratio can strongly affect catalytic performance [58]. The effects of DES-to-EB mass ratio from 1:3 to 1:8 on HMF production were evaluated in DES-H₂O (160 °C) for 60 min (Figure 6). When DES was not added to the reaction system (Control: the aqueous system), 175.8 mM HMF was generated from EB. In the DES-H₂O system containing different proportions of DES, the HMF concentration increased considerably as the weight ratio of DES and EB was from 1:3 to 1:5, and the HMF concentration increased from 229.4 to 261.2 mM. The concentration of HMF reached the maximum at DES:EB 1:5 (wt/wt), and then gradually decreased as the DES loading was

further increased. Consequently, DES:EB 1:5 (wt/wt) was the optimum ratio to produce HMF from EB.

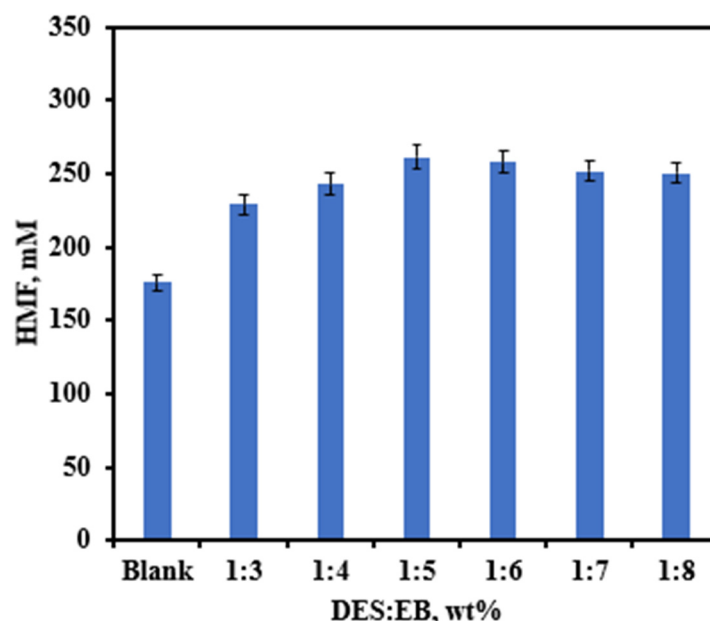


Figure 6. Influence of DES-to-EB mass ratio on HMF generation [All experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

2.4. Chemobiocatalytic Production of HMFA from EB in the MA:ChCl System

The transformation of waste bioresources into valuable chemicals has attracted increasing interest [59–64]. Integrating chemocatalysis and biocatalysis in a cascade process can improve the valorization of waste biomass; it is possible to promote the valorization of waste biomass [62]. This approach not only boosts the efficiency of chemocatalytic processes but also may reduce the need for intermediate-processing equipment [63]. In this study, the chemobiological transformation of expired beverages (EB) was first converted into HMF by chemocatalysis. The combination of chemo- and biological catalysis in a single reaction vessel has garnered attention as a means of enhancing valorization of biomass waste into valuable biobased compounds. In MA:ChCl (20.0 wt%), 261.2 mM HMF could be obtained from 33.3 g of EB at 160 °C for 60 min. After HMF production, the pH of the MA:ChCl-containing reaction mixture was adjusted to 8.0 using 1 M NaOH solution. Subsequently, the reaction mixture was diluted with sterile deionized water to reduce the DES concentration and decrease the HMF concentration to 215.5 mM before the addition of *E. coli* LF-PDC whole cells (0.1 g/mL, wet weight) and *D*-Ala (4 moles of *D*-Ala per mole of HMF). As evidenced in Figure 7, 215.5 mM of HMF was biologically aminated to yield 208.3 mM of HMFA after 12 h. This one-pot two-step process enabled the valorization of 33.3 g of EB into 208.3 mM of HMFA. This chemobiocatalytic process did not require purification of the HMF intermediate, which may simplify the process and reduce equipment requirements. In this research, HMF was acquired via chemical reaction in MA:ChCl using EB as a raw material, and then *E. coli* LF-PDC whole cells as biocatalysts were adopted to aminate HMF to efficiently produce HMFA.

Furthermore, mass flow from EB to HMFA was analyzed. Under the optimized conditions of 160 °C for 60 min at a DES-to-EB mass ratio of 1:5, 33.3 g EB was catalyzed to produce 1.732 g HMF, corresponding to the sugar-to-HMF mass conversion of 61.8%. After adjusting the pH of the HMF-containing reaction mixture to 8.5, 1.688 g HMFA was synthesized by adding *E. coli* LF-PDC whole cells and *D*-Ala, corresponding to the HMF-to-HMFA molar conversion of 96.6%. Overall, HMFA (1.688 g) was acquired from EB

(33.3 g) by a one-pot two-step process combining chemocatalysis and biocatalysis (Figure 8). Although EB-derived HMF could be directly subjected to biological amination without intermediate isolation, the crude reaction mixture contains DES, residual sugars, organic acids, and other soluble components derived from the beverage feedstock. Therefore, product purification represents an important consideration for future process development. After whole-cell biotransformation, recombinant cells can be removed by centrifugation or membrane filtration, followed by HMFA purification using adsorption resins, ion-exchange chromatography, or crystallization approaches. In parallel, recovery and reuse of the MA:ChCl DES should be investigated to improve process sustainability.

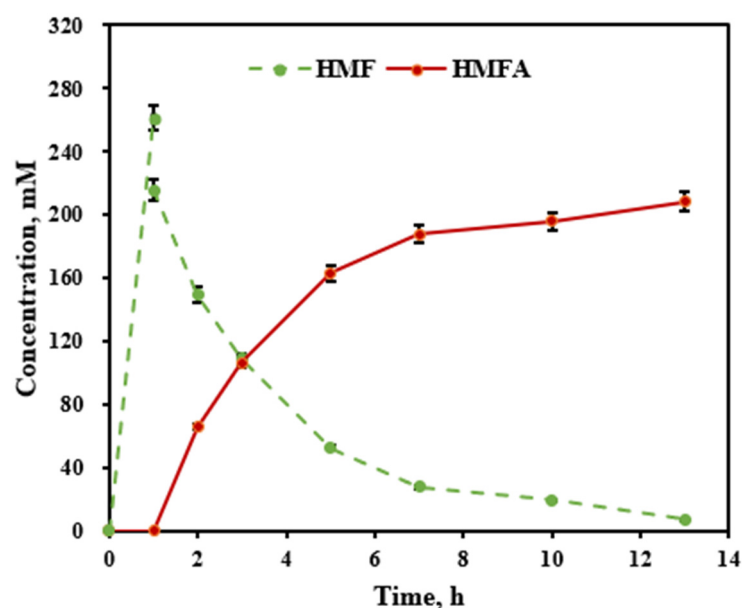


Figure 7. Time courses for converting EB into HMFA via chemobiocatalytic catalysis in the MA:ChCl system [All experiments were performed in triplicate, and data are shown as mean $\pm$ SD].

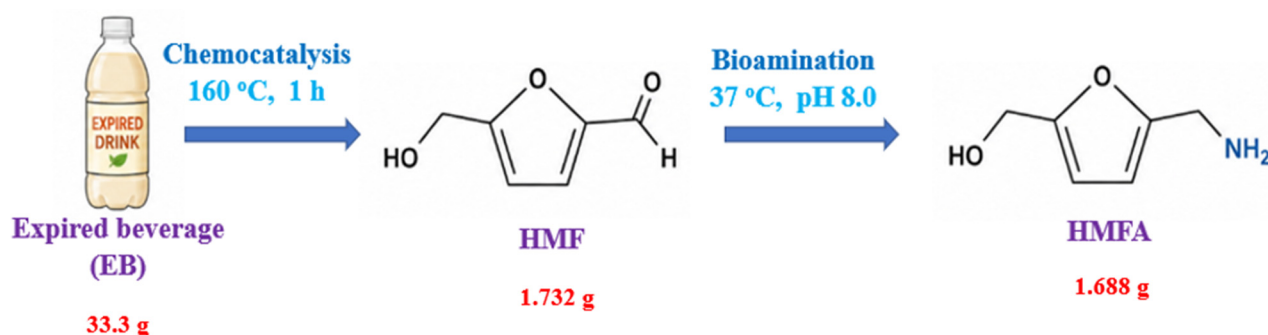


Figure 8. Mass flow from expired beverage (EB) to HMFA in MA:ChCl-H₂O via the chemobiological pathway.

Food waste is an important renewable feedstock for producing biobased chemicals [65,66]. Notably, sugar-sweetened beverages and fruit juices are important sources of C6 sugars (*D*-glucose and *D*-fructose) [10,67]. Recently, sugar-based beverage wastes have been investigated as potentially sustainable feedstocks, particularly for 5-HMF and its derivatives [68]. This study demonstrates the use of expired beverage (EB) waste as a feedstock for 5-HMF and HMFA production in a DES system. The EB-derived HMF could be efficiently aminated into HMFA through the *E. coli* LF-PDC whole-cell biocatalysis. EB might be chemobiologically transformed into HMFA via tandem transformation with MA:ChCl and *E. coli* LF-PDC whole cells. HMFA is an important nitrogen-containing

furan derivative with potential applications in curing agents, polymers, pharmaceutical intermediates, and agricultural chemicals [33]. Previous HMFA synthesis has mainly relied on ω -transaminase-catalyzed amination of purified HMF under mild conditions [34,35,64]. However, conventional transamination reactions usually require excessive amine donors because the accumulation of pyruvate shifts the equilibrium toward the substrate side [33], resulting in increased raw material consumption and reduced process economy [36,37]. To overcome this limitation, multienzyme cascade strategies have recently been developed. For example, co-expression of auxiliary enzymes capable of removing pyruvate has been demonstrated to enhance transamination efficiency and decrease amine donor consumption [38,39]. In previous studies, chemoenzymatic production of HMFA from waste-derived furan compounds was also explored. Wu et al. reported HMFA production from bread waste through a chemoenzymatic cascade process, demonstrating the feasibility of integrating waste valorization with biocatalysis [12]. Compared with these reported systems, the present study establishes a direct EB-to-HMFA conversion route by integrating MA:ChCl DES-mediated HMF production with LF-PDC whole-cell bioamination. The recombinant *E. coli* LF-PDC catalyst efficiently converted EB-derived HMF into HMFA while reducing the *D*-Ala requirement from HMF:*D*-Ala = 16:1 to 4:1. The final HMFA yield reached 96.6%, comparable to or higher than many previously reported HMF amination systems. Moreover, unlike conventional approaches requiring separation and purification of HMF before enzymatic transformation, the present one-pot two-step cascade process avoids intermediate isolation, reducing operational complexity and improving process integration. Therefore, this strategy provides a sustainable approach for converting food waste into value-added nitrogen-containing furan chemicals.

Valorization of waste in the beverage industry has attracted increasing attention in recent years. Although the present study demonstrates an efficient laboratory-scale route for HMFA production from expired beverage waste, downstream processing and catalyst recycling remain critical challenges for practical implementation. After biotransformation, HMFA can potentially be recovered through cell removal followed by adsorption-based purification or ion-exchange separation. The recombinant *E. coli* LF-PDC whole-cell catalyst may be recycled through cell recovery and regeneration, although immobilized whole-cell systems should be further explored to improve operational stability. In addition, recovery and reuse of the MA:ChCl DES and valorization of residual carbohydrates should be investigated to establish a closed-loop biorefinery process. Compared with conventional HMFA production from purified HMF, this waste-based chemobiocatalytic strategy reduces feedstock cost and amine-donor consumption; however, comprehensive techno-economic analysis and life-cycle assessment are required before industrial application. In the future, an integrated process involving EB pretreatment, catalysis, product separation, and wastewater treatment should be evaluated at a larger scale to determine its economic feasibility.

3. Materials and Methods

3.1. Materials

An expired beverage (EB) product was obtained from Pupu Supermarket in Wuhan, China, which contained sugar at 8.4% (mass ratio). Choline chloride (ChCl), malonic acid (MA), *D*-alanine (*D*-Ala), and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and SinoPharm Chemical Reagent Co., Ltd. (Shanghai, China).

3.2. Synthesis of DES Malonic Acid:Choline Chloride (MA:ChCl)

Choline chloride (ChCl) and malonic acid (MA) were mixed in the molar ratio of 1:2, and the mixture was placed in a 100 mL round-bottom flask. The mixture was stirred at 500 rpm and 60 °C until a clear, homogeneous liquid formed.

3.3. Transformation of EB into HMF

EB (33.3 g) was mixed with DES–water medium (MA:ChCl, 2–10 wt%) and reacted at 150–180 °C. in an autoclave (KLWX-JX, Shanghai Keli Equipment Co., Ltd., Shanghai, China) with stirring at 500 rpm. After 30–75 min, samples were removed, diluted and filtered, and then analyzed by HPLC. HMF yield was calculated as follows:

$$\text{HMF yield (wt\%)} = \frac{\text{HMF (M)} \times V \text{ (L)} \times 126.11}{\text{Sugar in EB (g)}} \times 100$$

where V indicates the volume of the reaction system, and 126.11 is the relative molecular mass of HMF.

3.4. Construction of Recombinant *E. coli* Co-Expressing LF and PDC

The LF gene [Leu (L) at AT187 was mutated to Phe (F)] and was generated by site-directed mutagenesis of the parent ω -transaminase gene (GenBank Accession No. XM_001209325). The LF gene fragments were amplified using LF-F (AATCTGCAGTGGGGTGATTTTGTTCGTGGTATGTTTGAAGCAGC) and AT-R (CGCCTGCGGTGGTACACATAAAGATTTTCATCACAACGATATGCCAG) primers. pRSFDuet-1 was amplified by pRSFDuet-F (AGCCAGGATCCGAATTCGAGC) and pRSFDuet-R (GTGGTGATGATGGTGATGGCTGC). The PDC gene was derived from *Zymomonas palmarum* T109 (IAM 14233, ATCC 51623), which was amplified by PDC-F (GGTACCTAAGGAGATATATCATGTATACCGTTGGCATGTATCTGGCAG) and PDC-R (GCTCGAATTCGGATCCTGCTTATGCCTGCGGTTTGCGACTATTTG). LF and PDC genes were independently amplified by PCR and subsequently assembled into a single pRSFDuet-1-based dual-expression plasmid using a T5 exonuclease-mediated homologous recombination strategy. Under the control of the T5 promoter, the PDC fragment was assembled with linearized pRSFDuet-LF using T5 exonuclease-dependent assembly (Figure S1, see Supplementary Materials). The resulting plasmid pRSFDuet-LF-PDC was introduced into *E. coli* BL21(DE3) via transformation, and the amplified fragments were subsequently verified by sequencing. Figure S2 (left) shows agarose gel electrophoresis results after colony PCR. The SDS-PAGE image confirmed the expression of proteins with apparent molecular masses of 36.2 kDa (LF) and 60.1 kDa (PDC) (Figure S2, right) (see Supplementary Materials).

The recombinant *E. coli* LF-PDC strain was cultured in LB medium containing Kanamycin (50 $\mu\text{g/mL}$) at 37 °C until the optical density at 600 nm reached approximately 0.6. Protein expression was induced by isopropyl β -D-1-thiogalactopyranoside (IPTG), and cultivation was continued under induction conditions. After induction, the culture was centrifuged ($8000 \times g$, 4 °C, 10 min). The resulting pellet (wet cells) was stored at -80 °C or used directly for subsequent whole-cell catalysis. The standard reaction mixture contained HMF, D-Ala, MA:ChCl DES- H_2O medium, and recombinant whole cells. No additional nutrient medium was added during biotransformation.

3.5. Analytical Method

HMF, HMFA, and 2,5-bis(hydroxymethyl)furan (BHMF) were quantified by a high-performance liquid chromatography system (Waters Nova-Pak, 3.9×150 mm, 4 μm) with a C18 column. The mobile phase consisted of methanol and water containing 0.1% trifluoroacetic acid at a volume ratio of 20:80. at a flow rate of 0.8 mL/min. HMFA and BHMF were detected at 210 nm, whereas HMF was detected at 254 nm. All experiments were performed in triplicate, and data are shown as mean $\pm$ SD.

4. Conclusions

The efficient and sustainable transformation of waste resources into high-value platform molecules has emerged as a strategic direction for advancing carbon-neutral biorefinery development. By constructing a recombinant *E. coli* strain co-expressing LF and PDC, the required loading of *D*-Ala as the amine donor was reduced to efficiently catalyze the conversion of EB-derived HMF into HMFA via biological amination. The PDC-catalyzed removal of pyruvate shifted the transamination equilibrium toward HMFA formation. Amine-donor consumption was reduced. An efficient one-pot two-step chemobiological transformation of EB was implemented to produce HMFA. Sugars in the EB were converted into HMF in MA:ChCl at a high concentration of up to 261.2 mM (160 °C, 60 min), and the EB-derived HMF could be biochemically aminated into HMFA. This hybrid strategy provided a new idea to solve the problem of food waste, providing a green and sustainable way for the efficient synthesis of HMFA. The present results provide a laboratory-scale basis for further process development. Future studies should focus on scale-up, catalyst and solvent recycling, downstream separation, wastewater treatment, and techno-economic assessment before industrial application can be considered.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal16080710/s1>, Figure S1. Construction of recombination *E. coli* expressing of LF and PDC; Figure S2. Verification of the recombinant pRSFDuet-LF-PDC construct and expression of LF and PDC proteins.

Author Contributions: Conceptualization, methodology, software, investigation, resources, data curation, writing—original draft: M.H. and H.Y.; Supervision, writing—review and editing: Y.-C.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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