Enhanced 2,5-Furandicarboxylic Acid (FDCA) Production in Raoultella ornithinolytica BF60 by Manipulation of the Key Genes in FDCA Biosynthesis Pathway

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The compound 2,5-furandicarboxylic acid (FDCA), an important bio-based monomer for the production of various polymers, can be obtained from 5-hydroxymethylfurfural (HMF). However, efficient production of FDCA from HMF via biocatalysis has not been well studied. In this study, we report the identification of key genes that are involved in FDCA synthesis and then the engineering of Raoultella ornithinolytica BF60 for biocatalytic oxidation of HMF to FDCA using its resting cells. Specifically, previously unknown candidate genes, *adhP3* and *alkR*, which were responsible for the reduction of HMF to the undesired product 2,5-bis(hydroxymethyl)furan (HMF alcohol), were identified by transcriptomic analysis. Combinatorial deletion of these two genes resulted in 85.7% reduction in HMF alcohol formation and 23.7% improvement in FDCA production (242.0 mM). Subsequently, an aldehyde dehydrogenase, AldH, which was responsible for the oxidation of the intermediate 5-formyl-2-furoic acid (FFA) to FDCA, was identified and characterized. Finally, FDCA production was further improved by overexpressing AldH, resulting in a 96.2% yield of 264.7 mM FDCA. Importantly, the identification of these key genes not only contributes to our understanding of the FDCA synthesis pathway in *R. ornithinolytica* BF60 but also allows for improved FDCA production efficiency. Moreover, this work is likely to provide a valuable reference for producing other furanic chemicals.

Keywords: 2,5-Furandicarboxylic acid, 2,5-bis(hydroxymethyl)furan, whole-cell biocatalysis, Raoultella ornithinolytica BF60, aldehyde dehydrogenase

Introduction

With the dwindling of fossil fuels, renewable raw sources of energy must be developed in prospective industries. Biomass is an abundant and cheap resource, and can be a potential candidate for the production of various chemicals [1]. 5-hydroxymethylfurfural (HMF), which can be obtained from the dehydration of carbohydrates derived from lignocellulose (Fig. 1A), is a promising platform chemical for the production of bio-based chemicals [2, 3]. For instance, HMF can be oxidized to 2,5-furandicarboxylic acid (FDCA), which has been regarded as a promising alternative to the oil-based terephthalic acid for the production of bio-based polyamides, polyesters, or polyurethanes [4]. As another example, poly(ethylene 2,5-furandicarboxylate) (PEF), the esterification product of ethylene glycol and FDCA, is comparable to poly(ethylene terephthalate) (PET) in the packaging materials for beverages, soft drinks, and water [5].

Currently, numerous chemical or photocatalysis methods via noble or non-noble metal catalysts have been reported for the selective oxidation of HMF to FDCA [6-10]. However, these methods are generally performed under high pressure/temperature and require additives. In comparison, biocatalytic reactions have many advantages, such as high specificity, mild reaction conditions, and a more attractive environmental footprint [11]. Numerous enzymatic reactions have been reported to synthesize...
FDCA from HMF by using various oxidases, such as HMF oxidase, fungal aryl-alcohol oxidase, nonspecific peroxigenase, chloroperoxidase, galactose oxidase M₅₅, periplasmic aldehyde oxidase, and lipase, or a combination of these enzymes [12–16]. Nonetheless, some limitations for these methods, such as the low substrate concentration (about 2 to 100 mM) used for enzymatic reactions, accumulation of byproducts, and uneconomical processes hinder practical applications. Compared with enzymatic catalysis, whole-cell conversions are more promising and possess advantages such as high stability, low cost (no need for enzyme purification), cofactor regenerating, reactive oxygen species degrading, and integration of multi-step reactions in one strain [17–19]. For instance, Koopman and co-workers, using recombinant Pseudomonas putida S12 harboring a HMF/furfural oxidoreductase (HmfH) as a whole-cell biocatalyst for FDCA production from HMF, obtained 30.1 g/l of FDCA with a yield of 97% at 144 h [20].

Raoultella ornithinolytica BF60 is a Gram-negative, nonmotile and facultative anaerobic organism which was isolated from the soil and belongs to the family Enterobacteriaceae [21]. It was initially classified as Klebsiella genus, and only recently separated as Raoultella genus based on new molecular analysis [22]. Although R. ornithinolytica is extensively found in insects, fishes, and aquatic environments, invasive infections by R. ornithinolytica were exceedingly rare in humans [23–25]. Moreover, Raoultella species have been investigated for biological production of some chemicals or enzymes, such as 2,3-butanediol [26], pullulanase [27], Tris-peptide complex [28], and polysaccharide-protein complex [29].

In our previous study, we engineered a R. ornithinolytica BF60 strain as a whole-cell biocatalyst for FDCA production from HMF by fine-tuning the FDCA synthesis pathway and reducing the formation of the byproduct 2,5-bis(hydroxymethyl)furan (HMF alcohol) via deletion of five genes (aldR, dkgA, akR, adhP1, and adhP2) guided by transcriptomic analysis, and 221.5 mM FDCA was obtained [30]. However, the HMF alcohol and 5-formyl-2-furoic acid (FFA) byproducts were accumulated at the end of the biocatalysis process when a higher concentration of HMF was used. The catalytic oxidation process of HMF into FDCA by engineered R. ornithinolytica BF60 is shown in Fig. 1B. In this work, guided by transcriptomic analysis, we further identified two other genes (adhP3 and alkR) that were responsible for HMF reduction to HMF alcohol, and then we significantly reduced the formation of HMF alcohol by a combinational deletion of these two genes. In addition, the aldH gene, which is responsible for the oxidation of the intermediate FFA to FDCA, was identified. Finally, the production of FDCA was further improved through overexpression of AldH in the engineered R. ornithinolytica BF60 strain. Thus, this work not only provides insight into the FDCA synthesis pathway in R. ornithinolytica BF60 but also offers guidance for the production of other similar furanic compounds.

![Fig. 1. The pathway of biomass conversion to HMF (A) and the synthetic pathway of FDCA from HMF in engineered R. ornithinolytica BF60 harboring HmfH (B).](image-url)
### Materials and Methods

#### Strains, Plasmids, and Chemicals

The details of the strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was used as host strain for gene cloning, and cultured at 37°C in Luria-Bertani (LB) medium (5 g/l yeast extract, 10 g/l tryptone, and 10 g/l NaCl) supplemented with appropriate antibiotics: 30 μg/ml of chloramphenicol and/or 50 μg/ml of kanamycin. *E. coli* BL21 (DE3) was used for protein purification, and cultured in LB or Terrific Broth (TB) medium (24 g/l yeast extract, 12 g/l tryptone, 4 ml/l glycerol, 17 mM KH₂PO₄ and 72 mM K₂HPO₄). The mutant *R. ornithinolytica* BF60 strain, ROBF60-DE14 (ΔadlRΔdkgAΔadlP1ΔadlP2::FRT), was constructed in a previous study [30], used as the starting strain and renamed as BFDE in this study.

HMF (98%) was obtained from Shanghai Rongli Chemical Technology Co., Ltd. (Shanghai, China). HMF acid (>98%) and HMF alcohol (>95%) were purchased from Matrix Scientific (Columbia, SC, USA). FFA (>97%) and FDCA (97%) were obtained from Sigma-Aldrich (USA). All other chemicals were purchased from commercial corporations at analytical grade.

#### Gene Manipulation

The gene encoding HmfH (ADE20408.1) from *Cupriavidus basilensis* HMF14 was synthesized and codon-optimized in our previous study [31]. The plasmids were transformed into *R. ornithinolytica* BF60 via electroporation as described previously [21]. The λ Red recombination system was used for gene deletion [32]. In short, the neighboring sequences (approximately 200 bp) of the target site were amplified using PCR with the corresponding primers (Table S1). Then, these two fragments and the FRT-Kan-FRT cassettes, amplified from plasmid pKD13, were joined via Fusion-PCR. After purification, the PCR products were transformed into a *R. ornithinolytica* BF60 strain that harbored the pKD46-Cm plasmid, and kan² transformants were selected. The plasmid pCP20 was introduced into a kan² clone to eliminate the

### Table 1. Strains and plasmids used in this study.

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<th>Strains or plasmids</th>
<th>Description</th>
<th>Source</th>
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<td><em>E. coli</em> BL21 (DE3)</td>
<td>Used for heterologous protein expression</td>
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<td>araC, araBp-Δαr-Δexo, Amp², Cm² (Chloramphenicol resistance gene was cloned into pkD46 at the BamHI site due to <em>R. ornithinolytica</em> BF60 was not sensitive to Ampicillin antibiotic)</td>
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<td>pCP20</td>
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Protein Purification and Enzyme Activity Assays

For protein purification, AldH was expressed as a C-terminal (His)_6-tagged protein in E. coli BL21 (DE3) and purified via HiTrap HP column (5 ml, GE Healthcare Bio-Sciences AB, Sweden) using the ÄKTA Start Purifier System (GE Healthcare Bio-Sciences AB, Sweden). In brief, E. coli BL21 (DE3) harboring the plasmid pET28a-aldH was cultured at 37°C for 12 h in LB medium supplemented with 30 μg/ml of kanamycin and 50 μg/ml of chloramphenicol. Then, the seed cultures were transferred (1%, v/v) into 30 ml of LB medium supplemented with 30 μg/ml of kanamycin and 50 μg/ml of chloramphenicol at 30°C for 12 h. Next, the seed cultures were transferred (1%, v/v) and grown in 250 ml of LB medium supplemented with 50 μg/ml of chloramphenicol at 30°C with shaking at 220 rpm. IPTG (final concentration of 0.2 mM) was added into the medium to induce enzyme expression when the OD_600 reached 0.6. After 24 h of incubation at 30°C, the cells were collected via centrifugation (8,000 × g for 20 min, 4°C). Then, the cell pellets were washed twice with 50 mM sodium phosphate buffer (pH 8.0), and resuspended to an OD_600 of 100 with the same buffer. Bioanalytical experiments were conducted in 100-ml Erlenmeyer flasks containing 10 ml of cell suspension and 0.5 g CaCO_3. HMF alcohol, HMF, HMF acid, FFA, and acidic products were measured from the concentration of HMF and FFA ranging from 0.005 to 10 mM and 0.005 to 2 mM, respectively.

Whole-Cell Biocatalyst Preparation

The recombinant R. ornithinolytica BF60 cells were cultured in 25 ml of LB medium supplemented with 30 μg/ml of chloramphenicol at 30°C with shaking at 220 rpm for 12 h. Next, the seed cultures were transferred (1%, v/v) and grown in LB medium supplemented with 50 μg/ml of chloramphenicol at 30°C with shaking at 220 rpm. IPTG (final concentration of 0.2 mM) was added into the medium to induce enzyme expression when the OD_600 reached 0.6. After 24 h of incubation at 30°C, the cells were collected via centrifugation (8,000 × g for 20 min, 4°C). Then, the cell pellets were washed twice with 50 mM sodium phosphate buffer (pH 8.0), and resuspended to an OD_600 of 100 with the same buffer. Bioanalytical experiments were conducted in 100-ml Erlenmeyer flasks containing 10 ml of cell suspension and 0.5 g CaCO_3. HMF alcohol, HMF, HMF acid, FFA, and acidic products were measured from the concentration of HMF and FFA ranging from 0.005 to 10 mM and 0.005 to 2 mM, respectively.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA in the cultured cells was extracted at 12 h in the absence or presence of 20 mM HMF in LB medium using the RNAprep Pure Cell/Bacteria Kit (TIANGEN, China) and quantified by the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The complementary DNA was synthesized with the PrimeScript RT reagent kit (TaKaRa, Japan) using the mRNA as template. qRT-PCR was conducted using SYBR Premix Ex Taq II (TaKaRa, Japan) on a LightCycler 480 II system (Roche, Switzerland). The internal standard 16S rRNA gene was used as the control for data normalization. Primers used for qRT-PCR are shown in Table S2.

Analytical Methods

The OD_600 of cell cultures was determined using a spectrophotometer (UVmini-1240, Shimadzu, Japan). An OD_600 of 1.0 corresponded to 0.45 ± 0.01 g cell dry weight (CDW)/L for R. ornithinolytica BF60. HMF alcohol, HMF, HMF acid, FFA, and FDCA were analyzed by HPLC (Agilent 1260 system) as previously described [31]. The yield of FDCA was defined as the ratio of the molarity of FDCA produced and the molarity of the theoretical FDCA amount, based on the HMF added. All experiments were conducted in triplicate, and the values are shown as means ± standard deviations.

Results

Reducing the Formation of HMF Alcohol by Combinatorial Gene Deletion

In our previous work, to reduce the HMF alcohol

from HMF, we analyzed the candidate genes which are responsible for HMF reduction to HMF alcohol in *R. ornithinolytica* BF60 by transcriptomics under HMF stress [30]. Although the five genes responsible for HMF alcohol formation have been deleted in *R. ornithinolytica* BF60, HMF alcohol was still detectable in the FDCA biosynthesis process. To further reduce the formation of HMF alcohol, five additional candidate genes (adhp3, adhp4, alkR, dkgB, and akR2) were chosen for investigating based on the transcriptomic data obtained in previous work [30]. Specifically, TE10_RS13910 encoding zinc-dependent alcohol dehydrogenase (AdhP3), TE10_RS01870 encoding zinc-binding alcohol dehydrogenase (AdhP4), TE10_RS16200 encoding alkene reductase (AlkR), TE10_RS06845 encoding 2,5-didehydrogluconate reductase B (DkgB), and TE10_RS07615 encoding aldo/keto reductase (AkR2) were upregulated by 1.57-, 2.94-, 4.54-, 1.25-, and 1.19-fold, respectively [30]. The sequences of these five genes of *R. ornithinolytica* BF60 are shown in Table S3. In addition, to validate these transcriptomic results, the expression of adhp3, adhp4, alkR, dkgB, and akR2 were quantified using qRT-PCR under HMF stress. As is shown in Fig. 2A, the qRT-PCR data were consistent with the transcriptomic results.

Based on the above observations, five mutant strains containing different gene deletions were constructed to investigate the effects of each gene on HMF alcohol formation. As shown in Fig. 2B, no remarkable changes in the formation of HMF alcohol were observed in strains with adhp4, alkR2, and dkgB deletions, when compared with the starting strain (Fig. 2B, BFDE), except for the strains with a deletion of either adhp3 or alkR, which showed a significant decrease in HMF alcohol (0.33 and 0.30 mM, respectively, vs. 1.47 mM in the BFDE strain). This indicated that both adhp3 and alkR were responsible for the reduction of HMF to HMF alcohol. Subsequently, as HMF alcohol production was not completely inhibited in the adhp3 or alkR deletion strain, we next constructed a BFDE-Δadhp3ΔalkR strain with a combinational deletion of both adhp3 and alkR genes (Fig. S1). As expected, the HMF alcohol concentration was reduced to a very low level (0.21 mM), although it was still generated (Fig. 2B). In addition, deletion of these two genes did not have important effects on cell growth (Fig. S2). Thus, the BFDE-Δadhp3ΔalkR strain with reduced HMF alcohol formation was chosen for further study.

**Improved FDCA Production by Using the BFDE-Δadhp3ΔalkR Strain**

HMF could be oxidized to form FDCA by the single HmfH enzyme [34]. Therefore, to enhance the production of FDCA, the plasmid pACYC-hmfH was introduced into the BFDE-Δadhp3ΔalkR strain, thereby generating the BFDE2-H strain. Subsequently, the performance of BFDE2-H and BFDE-H (BFDE strain harboring the plasmid pACYC-hmfH) strains for the production of FDCA was evaluated. Fig. 3 shows the time course of intermediate and FDCA.

![Fig. 2.](image-url)

**Fig. 2.** (A) Relative normalized expression of target genes in *R. ornithinolytica* BF60 strains were monitored by quantitative reverse transcription PCR (qRT-PCR). The expression levels were normalized and calculated relative to 16S rRNA. (B) HMF derivatives formation profile of mutant *R. ornithinolytica* BF60 strains corresponding to different gene deletions by flask culture. Culture conditions: The mutant *R. ornithinolytica* BF60 strains were cultured at 30°C in LB medium overnight, then the seed cultures were transferred (1%, v/v) and grown at 30°C in LB medium with 20 mM HMF for 12 h.
formation by the BFDE-H (Fig. 3A) and BFDE2-H (B) strains. As expected, less HMF alcohol was generated in the BFDE2-H strain compared with the BFDE-H strain, and the accumulation of HMF alcohol was eliminated at the end of the biotransformation process. In addition, 242.0 mM of FDCA with an 88.0% yield was obtained with the BFDE2-H strain, which was 31.2% higher compared to that with the BFDE-H strain (184.5 mM of FDCA with a 67.1% yield). In the bioconversion time course of HMF by the BFDE2-H strain (Fig. 3B), 5-hydroxymethyl-2-furoic acid (HMF acid) and FFA were accumulated as the main intermediates, and subsequently, HMF acid was converted to FFA and FDCA. Finally, the FFA remained as the only byproduct in the reaction mixture at the end of the bioconversion process, and thereby caused an impurity of FDCA.

**Fig. 3.** Biosynthesis of FDCA from HMF by BFDE-H (A) and BFDE2-H (B) strains.

**Fig. 4.** HMF derivatives formation profile of mutant R. ornithinolytica BF60 strains corresponding to different gene deletions by flask culture in the presence of HMF (A) and FFA (B).

Identification of Aldehyde Dehydrogenase for Catalysis of FFA Oxidation

To allow for further conversion of FFA to FDCA, one potential strategy is to overexpress enzymes that participate in FFA oxidation. However, the enzymes that participate in FFA oxidation in R. ornithinolytica BF60 have not been well characterized. Hence, in order to expand our understanding of the FFA conversion mechanism in R. ornithinolytica BF60, we re-analyzed the transcriptomic data that were obtained under HMF stress [30], because...
HMF and FFA both have an aldehyde group, and enzymes that catalyze HMF oxidation were also likely participating in FFA oxidation. Based on the transcriptomic data, two possible aldehyde dehydrogenase genes were chosen for further investigation. TE10_RS21820 encoding aldehyde dehydrogenase (AldH) and TE10_RS12240 encoding succinyl glutamate-semialdehyde dehydrogenase (AstD), were significantly upregulated by 14.81- and 288.65-fold, respectively, under HMF stress [30]. The sequences of these two genes in *R. ornithinolytica* BF60 are shown in Table S3.

Subsequently, the expression level of *aldH* and *astD* was validated by qRT-PCR under HMF stress. As shown in Fig. 2A, the qRT-PCR results were in accordance with the transcriptomic data. Next, the functions of these two aldehyde dehydrogenases were evaluated via gene deletion. Fig. 4A shows the shake flask culture results of the mutant *R. ornithinolytica* BF60 strains using HMF as the substrate. The accumulation of HMF acid was reduced in the *aldH* gene deletion strain, BFDE-ΔaldH, compared to the BFDE strain (0.15 vs. 1.13 mM). On the other hand, there was no significant difference in the accumulation of HMF acid between the BFDE and BFDE-ΔastD strains (1.13 vs. 1.14 mM). When using FFA as the substrate (Fig. 4B), less FDCA was generated in the BFDE-ΔaldH strain compared to the BFDE strain (0.16 vs. 0.60 mM), and more FFA remained (7.60 vs. 6.94 mM). In addition, deleting the *astD* gene had no significant effect on FDCA formation in the BFDE-ΔastD strain. Based on this observation, we thought that AldH might be responsible for HMF and FFA oxidation in *R. ornithinolytica* BF60.

To further confirm the functions of AldH, AldH was expressed as a (His)_6-tagged protein in *E. coli* BL21 (DE3) and purified for an *in vitro* catalysis reaction with HMF and FFA as the substrate. After AldH purification, a single band approximately 56.0 kDa was observed by SDS-PAGE analysis (Fig. 5). The *in vitro* reaction results showed that AldH could accept both HMF and FFA as substrates (Table 2). HMF was fully converted to HMF acid by AldH within 48 h when using NAD^+ or NADP^+ as the co-factor.

![Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified AldH.](image)

Lane M, protein marker; Lane 1, *E. coli* BL21 (DE3) harboring blank plasmid pET28a; Lane 2, *E. coli* BL21 (DE3) harboring plasmid pET28a-aldH; Lane 3, purified AldH.

In addition, FFA could also be oxidized by AldH to form FDCA, although the reaction activity was slower than that observed for HMF (Table 2). Kinetic parameters of AldH for HMF and FFA oxidation are presented in Table 3. AldH showed a relatively lower *K_\text{m}* value and catalytic efficiency (*K_\text{cat}*/ *K_\text{m}* for FFA compared to HMF when NAD^+ or NADP^+ were used as the co-factor. Overall, these results indicated that AldH was a promising candidate enzyme for the conversion of FFA to FDCA in *R. ornithinolytica* BF60.

### Overexpression of AldH to Improve FDCA Production

To generate a robust FDCA-producing strain, FFA, which accumulated in the BFDE2-H strain (Fig. 3B), should be converted to FDCA efficiently. Therefore, the plasmid pACYC-hmfH-aldH, which co-expressed the *hmfH* and *aldH* genes...
genes, was introduced into the BFDE-ΔadhP3ΔalkR strain, resulting in the BFDE2-HA strain. Subsequently, whole-cell biotransformation was performed with this recombinant strain. The time course of the HMF conversion and FDCA production was shown in Fig. 6. In the biotransformation process, FFA was detected in the early and middle stages, and was consumed later (after 120 h). In addition, more HMF acid was generated during the catalytic process by the BFDE2-HA strain (Fig. 6) compared to the BFDE2-H strain (Fig. 3B). Finally, due to the full conversion of FFA at the end of the bioconversion process, 264.7 mM of FDCA with a 96.2% yield was obtained at 144 h, and no HMF and other intermediates were detected at the end of the bioconversion.

**Discussion**

Furan aldehydes, such as HMF, are highly toxic to living microorganisms [35–37]. Therefore, various detoxification mechanisms, such as the reduction and/or oxidation to less toxic alcohol and acid forms, have evolved in many microorganisms [38, 39]. In analysis of the potential genes of *R. ornithinolytica* BF60 responsible for reducing HMF to HMF alcohol, two uncharacterized genes, *adhP3* and *alkR*, were identified. Sequence analysis showed that AdhP3 has a 44% identity with FurX, a characterized alcohol dehydrogenase from *Cupriavidus necator* JMP134 that has furfural reduction activity [40]. AlkR showed a 41% identity with an NADH: flavin oxidoreductase/NADH oxidase encoded by ZMO1885 gene in *Zymomonas mobilis* ZM4, which reduced phenolic aldehydes into phenolic alcohols [41]. After deleting the *adhP3* and *alkR* genes in the BFDE strain, HMF alcohol was still detectable in the cultures. This illustrates that some other endogenous nonspecific dehydrogenases which are responsible for HMF reduction should exist in the BFDE-ΔadhP3ΔalkR strain. In addition, these two identified genes, which possess HMF reduction activity, may be useful for the production of HMF alcohol or aromatic alcohols [42, 43], and were used to construct a furan aldehyde-resistant strain [44].

FDCA, a potential alternative to oil-based terephthalic acid, is often used for the production of bio-based polymers. However, impurities in the FDCA mixture, such as FFA, will terminate the chain reaction during the polymer production process. Although an aldehyde dehydrogenase 1 has been verified that possesses FFA oxidation activity in *R. ornithinolytica* BF60 [21], its performance was not satisfactory when overexpressed in BFDE2-H strain, since HMF acid was accumulated as the main intermediate and less FDCA was obtained (data not shown). In this study, we overexpressed the newly-identified aldehyde dehydrogenase AldH in the BFDE2-H strain, and no FFA was detected at the end of the reaction (Fig. 6). The AldH-overexpressing strain, BFDE2-HA, accumulated more HMF acid and less HMF alcohol compared to the BFDE2-H strain (without overexpression of AldH). We hypothesized that this may be because HMF was preferentially oxidized to HMF acid by AldH rather than being reduced to HMF alcohol. Through full conversion of the intermediate FFA to FDCA, 264.7 mM of FDCA was obtained with productivity of 0.29 g/l/h. In addition, FDCA productivity in *R. ornithinolytica* BF60 can be improved further by protein

**Table 3. Kinetic parameters of purified AldH.**

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<tr>
<th>Substrate</th>
<th>Co-factor</th>
<th>$K_m$ (mM)</th>
<th>$K_{cat}$ (min$^{-1}$)</th>
<th>$K_{cat}/K_m$ (mM$^{-1}$ min$^{-1}$)</th>
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<td>HMF</td>
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<td>HMF</td>
<td>NADP$^+$</td>
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<td>3.62 ± 0.17</td>
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<td>NAD$^+$</td>
<td>0.04 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>2.47 ± 0.09</td>
</tr>
<tr>
<td>FFA</td>
<td>NADP$^+$</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>2.13 ± 0.11</td>
</tr>
</tbody>
</table>

**Fig. 6.** Biosynthesis of FDCA from HMF by BFDE2-HA strain. Biotransformation conditions: 10 ml sodium phosphate buffer (50 mM, pH 8.0), $OD_{600} = 100$ microbial cells, 0.5 g CaCO$_3$, 220 rpm, 30°C. 50, 50, 50, 50, 25, 25, and 25 mM HMF were fed at 0, 12, 24, 36, 48, 60, and 72 h, respectively.
engineering of HmfH or endogenous dehydrogenase to strengthen intermediates conversion, like HMF acid and FFA.

Overall, we constructed a recombinant *R. ornithinolytica* BF60 strain that can efficiently synthesize FDCA from HMF without the accumulation of byproducts by the end of the biotransformation process. This result was achieved by a series of strategies, including decreasing the generation of the HMF alcohol byproduct through rational gene deletion, and eliminating FFA accumulation via overexpression of the newly-identified aldehyde dehydrogenase. The use of these identified genes, which are responsible for HMF reduction and FFA oxidation, may enable the biosynthesis of a variety of compounds derived from furan aldehydes, expanding the boundaries of biocatalysis or metabolic engineering.

**Acknowledgments**

This research was financially supported by the National Natural Science Foundation of China (31622001, 31671845, and 31600068), the Natural Science Foundation of Jiangsu Province (BK20160176), the Key Technologies R&D Program of Jiangsu Province, China (BE2016638) and the Fundamental Research Funds for the Central Universities (JUSRP51612A, JUSRP51713B).

**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


