J. Microbiol. Biotechnol. (2018), 28(12), 1999–2008
https://doi.org/10.4014/jmb.1808.08057

jmb

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

Haibo Yuan[1,2], Yanfeng Liu[1,2], Xueqin Lv[1], Jianghua Li[1,2], Guocheng Du[1], Zhongping Shi[2]*, and Long Liu[1]*

[1]*Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, 214122 Wuxi, P.R. China*
[2]*Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 214122 Wuxi, P.R. China*

Received: Aug 20, 2018
Revised: September 18, 2018
Accepted: September 27, 2018

First published online
October 10, 2018

**Corresponding authors*
Zhongping Shi
Phone: +86-510-85918292;
Fax: +86-510-85326276;
E-mail: zpshi@jiangnan.edu.cn
Long Liu
Phone: +86-510-85918312;
Fax: +86-510-85918309;
E-mail: longliu@jiangnan.edu.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

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

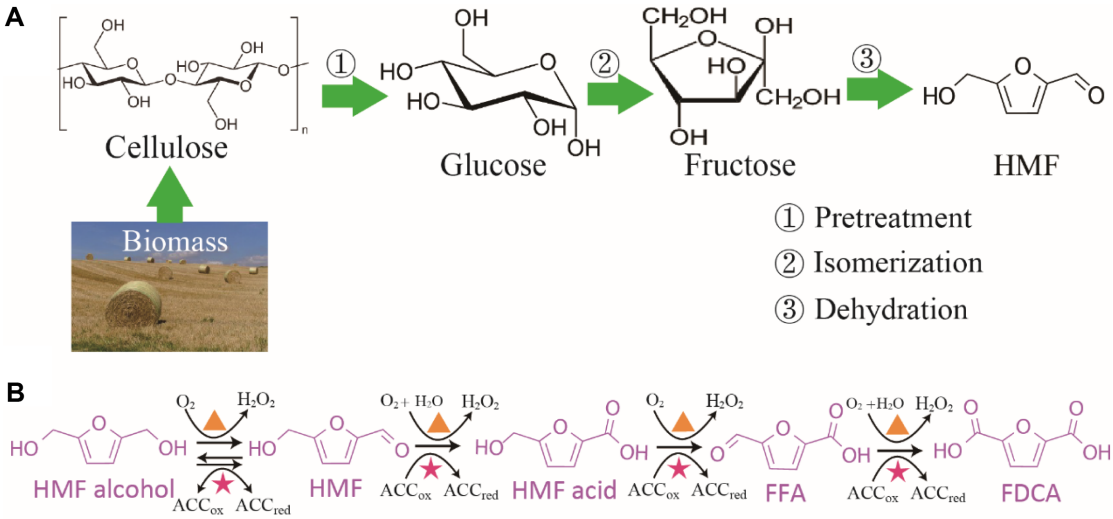

Fig. 1. The pathway of biomass conversion to HMF (**A**) and the synthetic pathway of FDCA from HMF in engineered *R. ornithinolytica* BF60 harboring HmfH (**B**).

Purple star (★), potential nonspecific dehydrogenases in *R. ornithinolytica* BF60; orange triangle (▲), HMF/furfural oxidoreductase (HmfH); ACC, acceptor, which is oxidized (ox) or reduced (red).

FDCA from HMF by using various oxidases, such as HMF oxidase, fungal aryl-alcohol oxidase, nonspecific peroxygenase, chloroperoxidase, galactose oxidase $M_{3\text{-}5}$, 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, non-motile 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.

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Description	Source
Strains		
E. coli JM109	Cloning strain	Takara, Ostu, Japan
E. coli BL21 (DE3)	Used for heterologous protein expression	Novagen
BFDE	*R. ornithinolytica* BF60_Δ*aldR*Δ*dkgA*Δ*akR*Δ*adhP1*Δ*adhP2*::FRT	[30]
BFDE-Δ*adhP3*	BFDE strain with *adhP3* gene deletion	This study
BFDE-Δ*adhP4*	BFDE strain with *adhP4* gene deletion	This study
BFDE-Δ*alkR*	BFDE strain with *alkR* gene deletion	This study
BFDE-Δ*akR2*	BFDE strain with *akR2* gene deletion	This study
BFDE-Δ*dkgB*	BFDE strain with *dkgB* gene deletion	This study
BFDE-Δ*adhP3*Δ*alkR*	BFDE strain with *adhP3* and *alkR* genes deletion	This study
BFDE-H	BFDE strain harboring plasmid pACYC-*hmfH*	This study
BFDE2-H	BFDE-Δ*adhP3*Δ*alkR* harboring plasmid pACYC-*hmfH*	This study
BFDE2-HA	BFDE-Δ*adhP3*Δ*alkR* harboring plasmid pACYC-*hmfH-aldH*	This study
Plasmids		
pKD46	Amp^R, *araC*, *araBp-*λ_γ*-*λ_β*-λexo* (red recombinase), temperature-sensitive replicon	[32]
pKD46-Cm	*araC*, *araBp-*λ_γ*-*λ_β*-λexo*, Amp^R, Cm^R (Chloramphenicol resistance gene was cloned into pKD46 at the BamHI site due to *R. ornithinolytica* BF60 was not sensitive to Ampicillin antibiotic)	This study
pKD13	Amp^R, Kan^R, template plasmid with FLP recognition target and Kan resistance gene	[32]
pCP20	Amp^R, Cm^R, FLP recombinase, temperature-sensitive replicon	[32]
pET28a	Kan^R, pBR322 replicon, *T7* promoter	Novagen
pET28a-*aldH*	Kan^R, pBR322 replicon, pET28a harboring *aldH* gene under the control of *T7* promoter	This study
pACYC	Cm^R, *lac I*, P15A replicon, constructed based on pACYCDuet-1 skeletons where the *T7* promoter was replaced with *trc* promoter	[30]
pACYC-*hmfH*	Cm^R, *lac I*, P15A replicon, pACYC harboring *hmfH* gene under the control of *trc* promoter and RBS11 at MCS1	[30]
pACYC-*hmfH-aldH*	Cm^R, *lac I*, P15A replicon, pACYC-*hmfH* harboring *aldH* gene under the control of *trc* promoter at MCS2	This study

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 KH_2PO_4 and 72 mM K_2HPO_4). The mutant *R. ornithinolytica* BF60 strain, ROBF60-DE14 (Δ*aldR*Δ*dkgA*Δ*akR*Δ*adhP1*Δ*adhP2*), 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 kan^R transformants were selected. The plasmid pCP20 was introduced into a kan^R clone to eliminate the

resistance marker at 40°C. The mutant strains were verified by agarose gel electrophoresis and DNA sequencing.

To construct pET28a-*aldH*, the primers 28a-AldH-F and 28a-AldH-R (Table S1) were used to amplify the *aldH* gene from the genomic DNA extract of *R. ornithinolytica* BF60, and then digested and ligated into the plasmid pET28a at NcoI-HindIII sites. As for pACYC-*hmfH-aldH* construction, the *aldH* gene was amplified with primers ACYC-AldH-F and ACYC-AldH-R (Table S1), which contained 20 bp flanking homologous sequences at NdeI and XhoI sites. Then, the *aldH* fragment was ligated to a linearized plasmid pACYC-*hmfH* (obtained by NdeI and XhoI enzymes restriction) via recombination using the ClonExpress II Kit (Vazyme, China), yielding plasmid pACYC-*hmfH-aldH*. The correct structure of plasmids were verified by restriction analysis and DNA sequencing.

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 HisTrap 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 50 μg/ml of kanamycin. Then the seed cultures were transferred (1%, v/v) and grown in 250 ml Erlenmeyer flasks containing 25 ml of TB medium supplemented with 50 μg/ml of kanamycin at 37°C and 220 rpm. When the optical density at 600 nm (OD_{600}) reached 0.6, the cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultured for 12 h at 25°C. The cells were harvested by centrifugation (8,000 ×*g* for 10 min, 4°C), washed twice with 20 mM sodium phosphate (pH 7.4), and resuspended in the same buffer. The cell extract was obtained by ultrasonication (VCX750, Sonics, CT, USA) and followed by centrifugation (14,000 ×*g* for 30 min, 4°C), and the supernatant was filtered through a 0.22 μm filter before loading onto the HisTrap HP column. The $(His)_6$-tagged AldH was eluted with 20 mM sodium phosphate (pH 7.4) containing 500 mM NaCl and 200 mM imidazole. The eluates containing AldH were desalted via a HiTrap Desalting column (GE Healthcare Bio-Sciences AB, Sweden) with 50 mM Tris-HCl (pH 7.5) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For SDS-PAGE analysis, the samples were prepared in denaturing buffer at 70°C for 10 min, then 10 μl samples were loaded on a 10% polyacrylamide gel (Invitrogen, NP0301BOX). After vertical electrophoresis on a mini-cell apparatus (Invitrogen, EI0001) at 120 V for 50 min, the gel was stained with 1‰ Coomassie Brilliant Blue (R-250) and destained with destaining solution (methanol, water, acetic acid = 5:4:1, v/v). The protein concentration was determined by the Bradford method using bovine serum albumin to produce the standard [33].

Enzymatic activity of AldH was determined by monitoring the increase in absorbance at 340 nm due to NAD(P)H formation. Kinetic parameters (K_m and K_{cat}) were estimated at 30°C in 50 mM Tris-HCl (pH 7.5) containing 1 mM NAD^+ or $NADP^+$ by varying 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 TB medium supplemented with 30 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. Biocatalytic experiments were conducted in 100-ml Erlenmeyer flasks containing 10 ml of cell suspension and 0.5 g $CaCO_3$. 50, 50, 50, 50, 25, 25, and 25 mM HMF were fed at 0, 12, 24, 36, 48, 60, and 72 h, respectively. The $CaCO_3$ was added for neutralizing the acidic products.

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

formation 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*, *akR2*, 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-Δ*adhP3*Δ*alkR* 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-Δ*adhP3*Δ*alkR* strain with reduced HMF alcohol formation was chosen for further study.

Improved FDCA Production by Using the BFDE-Δ*adhP3*Δ*alkR* Strain

HMF could be oxidized to form FDCA by the single

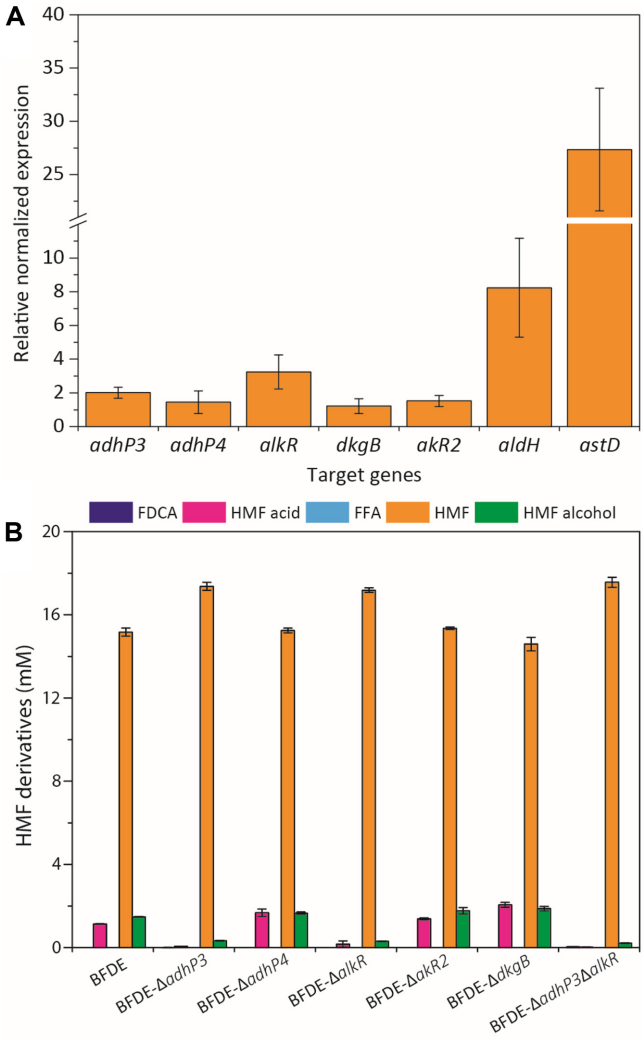

Fig. 2. (**A**) Relative normalized expression of target genes in *R. ornithinolytica* BF60 strains were monitored by quantitative reverse transcription PCR (qRT-PCR). The expression levels were normalized and calculated relative to 16S rRNA. (**B**) HMF derivatives formation profile of mutant *R. ornithinolytica* 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.

HmfH enzyme [34]. Therefore, to enhance the production of FDCA, the plasmid pACYC-*hmfH* was introduced into the BFDE-Δ*adhP3*Δ*alkR* 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

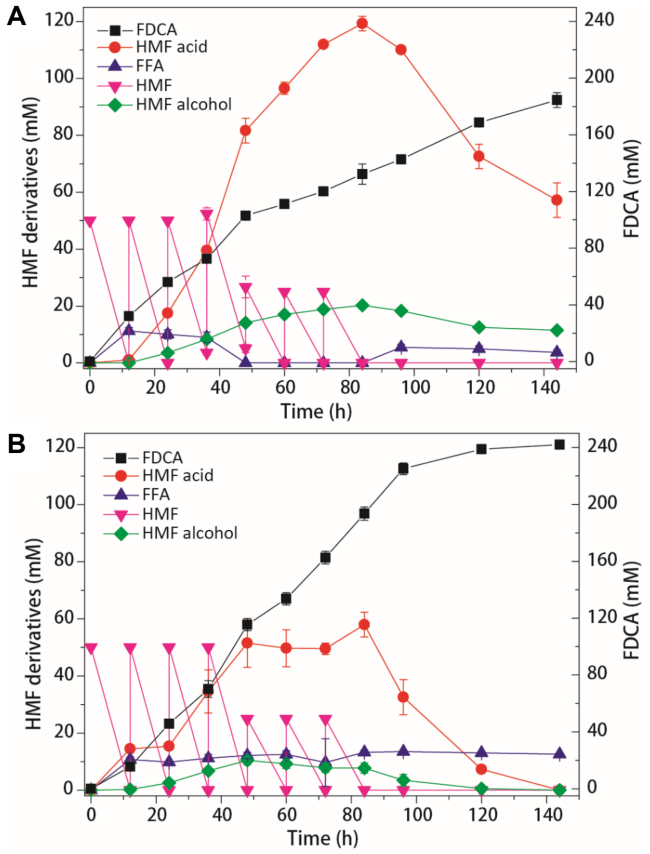

Fig. 3. Biosynthesis of FDCA from HMF by BFDE-H (A) and BFDE2-H (B) strains.

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.

formation by the BFDE-H (Fig. 3A) and BFDE2-H strains (Fig. 3B), with whole-cell biocatalysts using the HMF feeding strategy. 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.

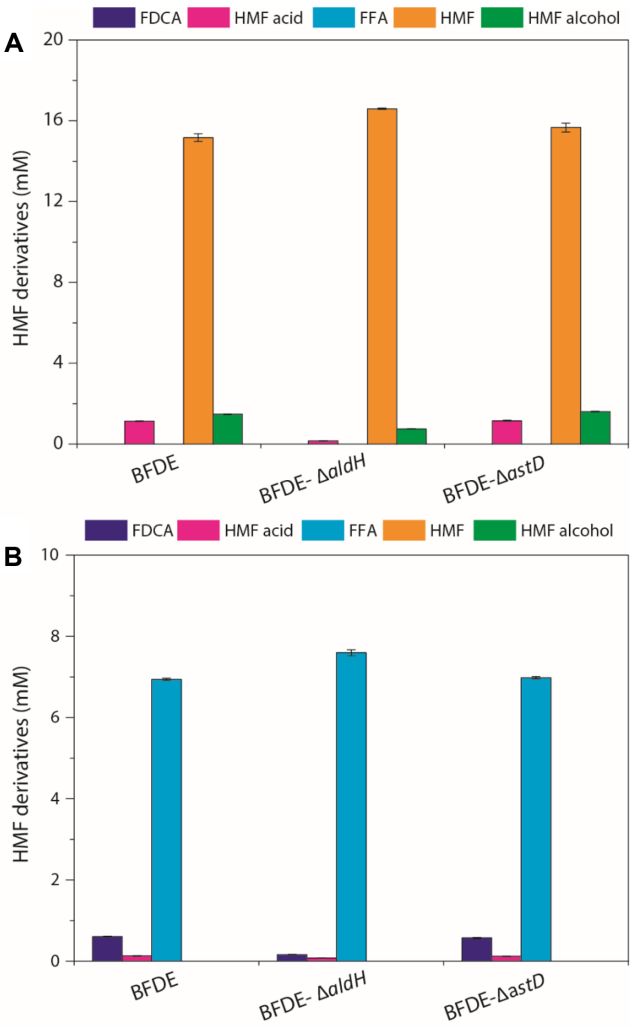

Fig. 4. HMF derivatives formation profile of mutant *R. ornithinolytica* BF60 strains corresponding to different gene deletions by flask culture in the presence of HMF (**A**) and FFA (**B**).

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 or 10 mM FFA for 12 h.

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 oxidization. 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 oxidization were also likely participating in FFA oxidization. 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-Δ*aldH*, 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-Δ*astD* strains (1.13 vs. 1.14 mM). When using FFA as the substrate (Fig. 4B), less FDCA was generated in the BFDE-Δ*aldH* 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-Δ*astD* 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.

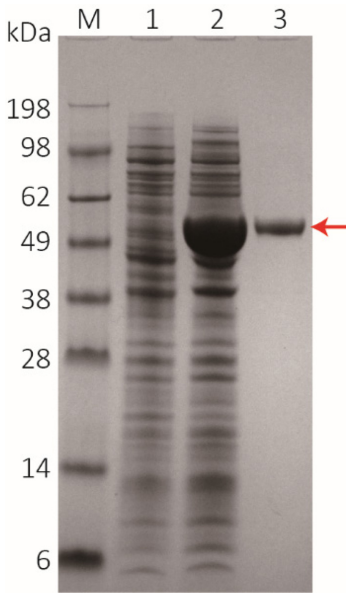

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified AldH.
Lane M, protein marker; Lane 1, *E. coli* BL21 (DE3) harboring blank plasmid pET28a; Lane 2, *E. coli* BL21 (DE3) harboring plasmid pET28a-*aldH*; Lane 3, purified AldH.

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_m value and catalytic efficiency (K_{cat}/K_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*

Table 2. HMF and FFA oxidation by purified AldH.

Entry[a]	Substrate	AldH (mg)	NAD^+ (mM)	$NADP^+$ (mM)	Conversion[b] (%)	HMF acid yield[b] (%)	FDCA yield[b] (%)
1	HMF	0.3	0.1	-[c]	100	100	0
2	HMF	0.3	-	0.1	100	100	0
3	FFA	0.3	0.1	-	10	0	10
4	FFA	0.3	-	0.1	9	0	9

[a]Reaction conditions: Final volume 2 ml, 50 mM pH 7.5 Tris-HCl, 1 mM substrate, 30°C, 220 rpm, 48 h.

[b]The conversion and yield were calculated by HPLC analysis.

[c]No addition.

Table 3. Kinetic parameters of purified AldH.

Substrate	Co-factor	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
HMF	NAD^+	0.17 ± 0.02	0.56 ± 0.04	3.29 ± 0.15
HMF	$NADP^+$	0.16 ± 0.03	0.58 ± 0.05	3.62 ± 0.17
FFA	NAD^+	0.04 ± 0.01	0.10 ± 0.03	2.47 ± 0.09
FFA	$NADP^+$	0.05 ± 0.01	0.10 ± 0.02	2.13 ± 0.11

genes, was introduced into the BFDE-Δ*adhP3*Δ*alkR* 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-Δ*adhP3*Δ*alkR* 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].

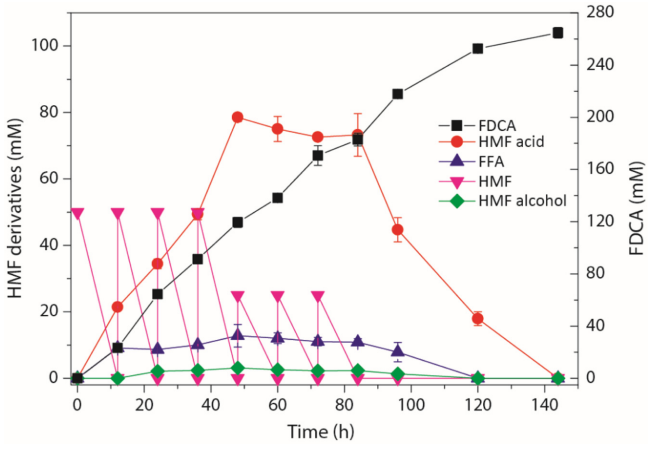

Fig. 6. Biosynthesis of FDCA from HMF by BFDE2-HA strain. Biotransformation conditions: 10 ml sodium phosphate buffer (50 mM, pH 8.0), OD_{600} = 100 microbial cells, 0.5 g $CaCO_3$, 220 rpm, 30°C. 50, 50, 50, 50, 25, 25, and 25 mM HMF were fed at 0, 12, 24, 36, 48, 60, and 72 h, respectively.

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

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

1. Zhou C-H, Xia X, Lin C-X, Tong D-S, Beltramini J. 2011. Catalytic conversion of lignocellulosic biomass to fine chemicals and fuels. *Chem. Soc. Rev.* **40:** 5588-5617.
2. Teong SP, Yi G, Zhang Y. 2014. Hydroxymethylfurfural production from bioresources: past, present and future. *Green Chem.* **16:** 2015-2026.
3. Mika LT, Cséfalvay E, Németh Á. 2018. Catalytic Conversion of Carbohydrates to Initial Platform Chemicals: Chemistry and Sustainability. *Chem. Rev.* **118:** 505-613.
4. Sousa AF, Vilela C, Fonseca AC, Matos M, Freire CSR, Gruter G-JM, *et al.* 2015. Biobased polyesters and other polymers from 2,5-furandicarboxylic acid: a tribute to furan excellency. *Polym. Chem.* **6:** 5961-5983.
5. Vilela C, Sousa AF, Fonseca AC, Serra AC, Coelho JFJ, Freire CSR, *et al.* 2014. The quest for sustainable polyesters–insights into the future. *Polym. Chem.* **5:** 3119-3141.
6. Zhang Z, Deng K. 2015. Recent advances in the catalytic synthesis of 2,5-furandicarboxylic acid and its derivatives. *ACS Catal.* **5:** 6529-6544.
7. Wang K-F, Liu C-l, Sui K-y, Guo C, Liu C-Z. 2018. Efficient catalytic oxidation of 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid by magnetic laccase catalyst. *Chembiochem.* **19:** 654-659.
8. Han X, Li C, Liu X, Xia Q, Wang Y. 2017. Selective oxidation of 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid over MnO_x–CeO_2 composite catalysts. *Green Chem.* **19:** 996-1004.
9. Xu S, Zhou P, Zhang Z, Yang C, Zhang B, Deng K, *et al.* 2017. Selective oxidation of 5-hydroxymethylfurfural to 2,5-furandicarboxylic acid using O_2 and a photocatalyst of co-thioporphyrazine bonded to g-C_3N_4. *J. Am. Chem. Soc.* **139:** 14775-14782.
10. Nam D-H, Taitt BJ, Choi K-S. 2018. Copper-based catalytic anodes to produce 2,5-furandicarboxylic acid, a biomass-derived alternative to terephthalic acid. *ACS Catal.* **8:** 1197-1206.
11. Nestl BM, Hammer SC, Nebel BA, Hauer B. 2014. New generation of biocatalysts for organic synthesis. *Angew. Chem. Int. Ed.* **53:** 3070-3095.
12. Karich A, Kleeberg SB, Ullrich R, Hofrichter M. 2018. Enzymatic preparation of 2,5-furandicarboxylic acid (FDCA)—a substitute of terephthalic acid—by the joined action of three fungal enzymes. *Microorganisms* **6:** 5.
13. McKenna SM, Mines P, Law P, Kovacs-Schreiner K, Birmingham WR, Turner NJ, *et al.* 2017. The continuous oxidation of HMF to FDCA and the immobilisation and stabilisation of periplasmic aldehyde oxidase (PaoABC). *Green Chem.* **19:** 4660-4665.
14. McKenna SM, Leimkühler S, Herter S, Turner NJ, Carnell AJ. 2015. Enzyme cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem. *Green Chem.* **17:** 3271-3275.
15. Carro J, Ferreira P, Rodríguez L, Prieto A, Serrano A, Balcells B, *et al.* 2015. 5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. *FEBS J.* **282:** 3218-3229.
16. Dijkman WP, Groothuis DE, Fraaije MW. 2014. Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid. *Angew. Chem. Int. Ed.* **53:** 6515-6518.
17. Wachtmeister J, Rother D. 2016. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. *Curr. Opin. Biotechnol.* **42:** 169-177.
18. Ladkau N, Schmid A, Bühler B. 2014. The microbial cell—functional unit for energy dependent multistep biocatalysis. *Curr. Opin. Biotechnol.* **30:** 178-189.
19. Schrewe M, Julsing MK, Bühler B, Schmid A. 2013. Whole-cell biocatalysis for selective and productive C–O functional group introduction and modification. *Chem. Soc. Rev.* **42:** 6346-6377.

20. Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ. 2010. Efficient whole-cell biotransformation of 5-(hydroxymethyl) furfural into FDCA, 2,5-furandicarboxylic acid. *Bioresour. Technol.* **101:** 6291-6296.
21. Hossain GS, Yuan H, Li J, Shin H-d, Wang M, Du G, *et al.* 2017. Metabolic engineering of *Raoultella ornithinolytica* BF60 for production of 2,5-furandicarboxylic acid from 5-hydroxymethylfurfural. *Appl. Environ. Microbiol.* **83:** e02312-02316.
22. Drancourt M, Bollet C, Carta A, Rousselier P. 2001. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int. J. Syst. Evol. Microbiol.* **51:** 925-932.
23. Sun F, Yin Z, Feng J, Qiu Y, Zhang D, Luo W, *et al.* 2015. Production of plasmid-encoding NDM-1 in clinical *Raoultella ornithinolytica* and *Leclercia adecarboxylata* from China. *Front. Microbiol.* **6:** 458.
24. Haruki Y, Hagiya H, Sakuma A, Murase T, Sugiyama T, Kondo S. 2014. Clinical characteristics of *Raoultella ornithinolytica* bacteremia: A case series and literature review. *J. Infect. Chemother.* **20:** 589-591.
25. Solak Y, Gul EE, Atalay H, Genc N, Tonbul HZ. 2011. A rare human infection of *Raoultella ornithinolytica* in a diabetic foot lesion. *Ann. Saudi. Med.* **31:** 93-94.
26. Kim T, Cho S, Lee S-M, Woo HM, Lee J, Um Y, *et al.* 2016. High production of 2,3-butanediol (2,3-BD) by *Raoultella ornithinolytica* B6 via optimizing fermentation conditions and overexpressing 2,3-BD synthesis genes. *PLoS One* **11:** e0165076.
27. Hii LS, Rosfarizan M, Ling TC, Ariff AB. 2012. Statistical optimization of pullulanase production by *Raoultella planticola* DSMZ 4617 using sago starch as carbon and peptone as nitrogen sources. *Food Bioproc. Tech.* **5:** 729-737.
28. Fiołka MJ, Grzywnowicz K, Rzymowska J, Lewtak K, Szewczyk R, Mendyk E, *et al.* 2015. Antitumour and apoptotic effects of a novel Tris-peptide complex obtained after isolation of *Raoultella ornithinolytica* extracellular metabolites. *J. Appl. Microbiol.* **118:** 1357-1369.
29. Fiołka MJ, Lewtak K, Rzymowska J, Grzywnowicz K, Hułas-Stasiak M, Sofińska-Chmiel W, *et al.* 2013. Antifungal and anticancer effects of a polysaccharide–protein complex from the gut bacterium *Raoultella ornithinolytica* isolated from the earthworm *Dendrobaena veneta*. *Pathog. Dis.* **69:** 49-61.
30. Yuan H, Liu Y, Li J, Shin H-d, Du G, Shi Z, *et al.* 2018. Combinatorial synthetic pathway fine-tuning and comparative transcriptomics for metabolic engineering of *Raoultella ornithinolytica* BF60 to efficiently synthesize 2,5-furandicarboxylic acid. *Biotechnol. Bioeng.* **115:** 2148-2155.
31. Yuan H, Li J, Shin H-d, Du G, Chen J, Shi Z, *et al.* 2018. Improved production of 2,5-furandicarboxylic acid by overexpression of 5-hydroxymethylfurfural oxidase and 5-hydroxymethylfurfural/furfural oxidoreductase in *Raoultella ornithinolytica* BF60. *Bioresour. Technol.* **247:** 1184-1188.
32. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97:** 6640-6645.
33. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
34. Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ. 2010. Identification and characterization of the furfural and 5-(hydroxymethyl)furfural degradation pathways of *Cupriavidus basilensis* HMF14. *Proc. Natl. Acad. Sci. USA* **107:** 4919-4924.
35. Wierckx N, Koopman F, Ruijssenaars HJ, de Winde JH. 2011. Microbial degradation of furanic compounds: biochemistry, genetics, and impact. *Appl. Microbiol. Biotechnol.* **92:** 1095-1105.
36. Ishida Y, Nguyen TTM, Izawa S. 2017. The yeast *ADH7* promoter enables gene expression under pronounced translation repression caused by the combined stress of vanillin, furfural, and 5-hydroxymethylfurfural. *J. Biotechnol.* **252:** 65-72.
37. Song H-S, Jeon J-M, Choi YK, Kim J-Y, Kim W, Yoon J-J, *et al.* 2017. L-Glycine alleviates furfural-induced growth inhibition during isobutanol production in *Escherichia coli*. *J. Microbiol. Biotechnol.* **27:** 2165-2172.
38. Wang X, Gao Q, Bao J. 2015. Transcriptional analysis of *Amorphotheca resinae* ZN1 on biological degradation of furfural and 5-hydroxymethylfurfural derived from lignocellulose pretreatment. *Biotechnol. Biofuels.* **8:** 136.
39. Tsuge Y, Hori Y, Kudou M, Ishii J, Hasunuma T, Kondo A. 2014. Detoxification of furfural in *Corynebacterium glutamicum* under aerobic and anaerobic conditions. *Appl. Microbiol. Biotechnol.* **98:** 8675-8683.
40. Li Q, Metthew Lam LK, Xun L. 2011. *Cupriavidus necator* JMP134 rapidly reduces furfural with a Zn-dependent alcohol dehydrogenase. *Biodegradation* **22:** 1215-1225.
41. Yi X, Gu H, Gao Q, Liu ZL, Bao J. 2015. Transcriptome analysis of *Zymomonas mobilis* ZM4 reveals mechanisms of tolerance and detoxification of phenolic aldehyde inhibitors from lignocellulose pretreatment. *Biotechnol. Biofuels* **8:** 153.
42. Xu Z-H, Cheng A-D, Xing X-P, Zong M-H, Bai Y-P, Li N. 2018. Improved synthesis of 2,5-bis(hydroxymethyl)furan from 5-hydroxymethylfurfural using acclimatized whole cells entrapped in calcium alginate. *Bioresour. Technol.* **262:** 177-183.
43. Koma D, Yamanaka H, Moriyoshi K, Ohmoto T, Sakai K. 2012. Production of aromatic compounds by metabolically engineered *Escherichia coli* with an expanded shikimate pathway. *Appl. Environ. Microbiol.* **78:** 6203-6216.
44. Miller EN, Jarboe LR, Yomano LP, York SW, Shanmugam KT, Ingram LO. 2009. Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. *Appl. Environ. Microbiol.* **75:** 4315-4323.