Metabolic engineering of *Escherichia coli* for producing adipic acid through the reverse adipate-degradation pathway

Mei Zhao\(^{a,b}\), Dixuan Huang\(^{a,b}\), Xiaojuan Zhang\(^{a}\), Mattheos A.G. Koffas\(^{c,d}\), Jingwen Zhou\(^{a,b,∗}\), Yu Deng\(^{a,b,∗}\)

\(^{a}\) National Engineering Laboratory for Cereal Fermentation Technology (NELCF), Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China
\(^{b}\) School of Biotechnology, Jiangnan University, 1800 Lihu Rd, Wuxi, Jiangsu 214122, China
\(^{c}\) Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
\(^{d}\) Department of Biological Sciences, Rensselaer Polytechnic Institute, Troy NY 12180, USA

**Abstract**

Adipic acid is an important dicarboxylic acid mainly used for the production of nylon 6–6 fibers and resins. Previous studies focused on the biological production of adipic acid directly from different substrates, resulting in low yields and titer. In this study, a five-step reverse adipate-degradation pathway (RADP) identified in *Thermohalophila fusca* has been reconstructed in *Escherichia coli* BL21 (DE3). The resulting strain (Mad136) produced 0.3 g L\(^{-1}\) adipic acid with a 11.1% theoretical yield in shaken flasks, and we confirmed that the step catalyzed by 5-Carboxy-2-pentenoyl-CoA reductase (*Tfu_1647*) as the rate-limiting step of the RADP. Overexpression of *Tfu_1647* by pTrc99A carried by strain Mad146 produced with a 49.5% theoretical yield in shaken flasks. We further eliminated pathways for major metabolites competing for carbon flux by CRISPR/Cas9 and deleted the succinate-CoA ligase gene to promote accumulation of succinyl-CoA, which is the precursor for adipic acid synthesis. The final engineered strain Mad123146, which could achieve 93.1% of the theoretical yield in the shaken flask, was able to produce 68.0 g L\(^{-1}\) adipic acid by fed-batch fermentation. To the best of our knowledge, these results constitute the highest adipic acid titer reported in *E. coli*.

### 1. Introduction

Adipic acid is a dicarboxylic acid that has extensive applications in the chemical industry, medicine, and lubricant manufacturing, and is mainly used for the production of nylon 6–6 fibers and resins (Deng et al., 2016; Polen et al., 2013; Vardon et al., 2015; Yu et al., 2014). Additionally, adipic acid is one of 12 bio-based chemicals with the greatest market value from renewable substrates reported by the United States Department of Energy (Werpy and Petersen, 2004). The adipic acid market yields ~4676,850,000 US dollars annually, with 2850,000 US tons produced globally, which has increased ~4.1% annually (Polen et al., 2013).

Adipic acid is currently produced from feedstocks derived from petroleum, specifically by oxidation of a mixture of cyclohexanone and cyclohexanol (KA oils) catalyzed by nitric acid (Niu et al., 2002; Polen et al., 2013; Sato et al., 1998). However, this chemical synthesis results in high levels of pollution and greenhouse gas emissions (U.S. Environmental Protection Agency, 2011). Therefore, there are great incentives to discover alternative approaches allowing for renewable and affordable adipic acid production. Recently, with the rapid development of biotechnology, the synthesis of adipic acid via metabolic engineering and synthetic biology approaches has attracted increased attention (Cheong et al., 2016; Deng et al., 2016; Polen et al., 2013; Vardon et al., 2015; Yu et al., 2014). A two-stage method consisting of biological synthesis and chemical reactions was established involving glucose fermentation to cis,cis-muconic acid (ccMA) (Jung et al., 2015; Weber et al., 2012; Wu et al., 2004; Wu et al., 2006) and ccMA hydrogenation to adipic acid catalyzed by 10% Pt (Draths and Frost, 1994; Niu et al., 2002). However, the chemical conversion of ccMA to adipic acid is expensive, environmentally damaging, and results in low yield (0.17 g adipic acid per g glucose) (Vardon et al., 2015). Therefore, there is a need to discover alternative methods for the direct synthesis of adipic acid from renewable substrates, such as glucose and glycerol.

Yu et al. constructed an artificial adipic acid synthesis pathway (Yu et al., 2014) involving β-ketoadipyl-CoA thiolase (PaaJ) from *Escherichia coli*, 3-hydroxyacyl-CoA reductase (*PaaH1*) from *Ralstonia eutropha*, enoyl-CoA hydratase (Ech) from *R. eutropha* H16, trans-enoxy-CoA reductase (*Ter*) from *Euglena gracilis*, butyryl kinase (Buk1) from

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Clostridium acetobutylicum, phosphate butyryltransferase (Ptb) from C. acetobutylicum in E. coli, producing ~0.0007 g L\(^{-1}\) adipic acid from glucose. Based on this pathway, Cheong et al. engineered E. coli MG1655 (ΔldhA, ΔapoB, Δpta, ΔadhE, and ΔsucD) to produce 2.5 g L\(^{-1}\) adipic acid from 50 g L\(^{-1}\) glycerol (9.6% theoretical yield)(Cheong et al., 2016). However, the adipic acid titers were far from those required for industrial application.

Previously, there were no native adipic acid synthesis pathways determined in microorganisms. However, Deng et al. reported the existence of a native adipic acid synthesis pathway in the thermophilic actinobacterium Thermobifida fusca (Deng and Mao, 2015) and identified it as a reverse adipate-degradation pathway (RADP) that included five enzymes (Fig. 1): Tfu_0875 (β-ketothiolase), Tfu_2399 (3-hydroxyacyl-CoA dehydrogenase), Tfu_0067 (3-hydroxyacyl-CoA dehydrogenase), Tfu_1647 (5-Carboxy-2-pentenoyl-CoA reductase) and Tfu_2576-7 (adipoyl-CoA synthetase). They reported that T. fusca B6 produced 2.23 g L\(^{-1}\) adipic acid from 50 g L\(^{-1}\) glucose; however, due to the lack of tools available for genetically engineering T. fusca, it was difficult to increase adipic acid titer and yield.

Here, we constructed the T. fusca RADP in E. coli for adipic acid production. After we confirmed the rate-limiting step (Tfu_1647) in the RADP, the corresponding enzyme was over-expressed for increasing the adipic acid production. Additionally, pathways for the metabolites competing for carbon flux were eliminated by CRISPR/Cas9, and the concentration of the precursor of adipic acid, succinyl-CoA, was increased by deleting the succinyl-CoA synthetase alpha subunit (sucD) gene. Furthermore, we optimized fermentation conditions for the engineered strain to achieve a high titer of adipic acid. Our findings represent the first example of expressing a native microorganism RADP pathway in E. coli, resulting in both high yield and titer of the target product.

2. Materials and methods

2.1. Bacterial strains and cultivation

The strains used in this study are listed in Supplementary Table 1. The E. coli strains were grown on M9, Lysogeny broth (LB), super optimal broth (SOB), super optimal broth with catabolite repression (SOC), 3-(N-morpholino)propanesulfonic acid broth (MOPS), and terrific broth (TB) (the media recipes are shown in Supplementary methods) with addition of chemicals as desired in 250 mL shake flasks with 200 rpm agitation on 4 g L\(^{-1}\) glucose. Gene expression was induced by IPTG at various final concentrations. All genes from organisms other than E. coli were codon optimized for E. coli by Geneviz (Suzhou, China). The production of adipic acid by E. coli in a 5-L bioreactor was conducted at 37 °C for culturing cells and at 30 °C for inducing gene expression with 1 vvm aeration and 400 rpm agitation with 1 mM IPTG.

For the fed-batch fermentation, a 5-L bioreactor (Baoxing, Shanghai, China) was used to conduct the fed-batch fermentation for the final strain. The two-stage fermentation strategy was employed in this study. The initial culturing temperature was 37 °C. During the first stage, 2% of the working volume seed culture was inoculated to the bioreactor and fermentation was initiated with 20 g L\(^{-1}\) glucose. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the medium once OD (600 nm) reached 0.6 after inoculation and then the culturing temperature was decreased to 30 °C. During the second stage, desired amount of 500 g L\(^{-1}\) glycerol added to the bioreactor. The pH was maintained at 7.0 by online measurement using a pH sensor and addition of 2 mol L\(^{-1}\) NaOH. The aeration and agitation rates varied according to different conditions.

2.2. Plasmid construction

Plasmids and primers used in this study are listed in Supplementary Table 1 and Supplementary Table 2. The synthesized genes are shown in Supplementary Table 3. Tfu_0875 was synthesized and ligated into plasmid pUC57-1 by Geneviz (Suzhou, China) and amplified by primers pUC57-1F (EcoR I) and pUC57-1R (Hind III). The PCR product and pRFSDuet-1 plasmid were digested by EcoR I and Hind III, and Tfu_0875 was ligated into pRFSDuet-1 using the T4 DNA ligase. The ligation product was introduced into E. coli JM109 for screening by colony PCR and Sanger sequencing. The final plasmid was named pRFSF-Tfu_0875. Tfu_2399 was amplified by primers pUC57-2F (Bgl II) and pUC57-2R (Kpn I) and ligated into pRFSF-Tfu_0875, which was digested with Bgl II and Kpn I to form pAD1. Plasmids pAD3, pAD6, pAD9, pAD10, and pAD11 were constructed according to the same protocol described previously, and the primers used are listed in Supplementary Table 2. Tfu_0067 and Tfu_1647 were amplified by the primers pUC57-3R and pUC57-3F and pUC57-4R and pUC57-4F from pUC57-3 and pUC57-4, respectively. PTrc99A was linearized by Nco I and Hind III restriction digest, and primers pAD4-0067F and pAD4-1647R contained 39-nt sequences homologous to the upstream and downstream regions of pTrc99A at the Nco I and Hind III sites, with the two genes linked by an RBS (AAGAGGATATATAT). Plasmid pAD4 was constructed using Gibson assembly methods (Gibson et al., 2009) and transformed into E. coli JM109 competent cells. The remaining plasmids developed in this study were constructed following the same protocol described above and are described in Supplementary Table 1.
2.3. Gene deletions in *E. coli* BL21 (DE3) cells by CRISPR-Cas9

The general procedure of deleting genes in *E. coli* BL21 (DE3) was described previously by Jiang et al. (Jiang et al., 2015). The plasmid pCas harbored Cas9 and single-guide RNA (sgRNA) targeting plasmid pTargetF (Jiang et al., 2015). The pTargetF harboring sgRNA (constitutive expression) targeting the gene to be deleted and donor DNA were transformed into cells with pCas, and 10 mM arabinose was added to induce λ-Red on pCas in order to promote homologous recombination. The mutant lacking homologous recombination by the donor DNA did not survive, whereas the one with the donor DNA replacing the target gene was able to grow on the agar plate. IPTG (0.5 mM) was used to induce the expression of sgRNA, which cut the targeting pTargetF using Cas9. The pCas was eliminated upon heating the culture to ∼42 °C. The sgRNAs targeting *sucD*, *atoB*, and *ldhA* genes are shown in Supplementary Table 3.

2.4. GC-MS identification of adipic acid

Adipic acid in the fermentation broth was extracted with ethyl acetate. Samples were vacuum dried after re-dissolving sample with acetonitrile, and extracted using silylation reagent at a ratio of N,O-bis(trimethylsilyl)trifluoroacetamide to trimethylchlorosilane of 99:1 and analyzed by GC-MS (Cheong et al., 2016) using a GCMS-QP2010 ultra mass selective detector (Shimadzu, Kyoto, Japan) at 70 eV electron impact to record mass spectra. The scan ranged from 50 m/z to 450 m/z, and the detector gain was set to 1.67 kV. The interface, ion source, and quadrupole mass analyzer were maintained at 220 °C, 200 °C, and 240 °C, respectively. The GC conditions were as follows: initial temperature was 50 °C, with a 1-min isothermal time; the heating program was 8 °C per min up to 180 °C without isothermal time, followed by 10 °C per min up to 240 °C, with an isothermal time of 5 min; and the carrier gas was helium with a constant flow rate of 1 mL min⁻¹. GC-MS software (Shimadzu Corporation Technologies, Kyoto Japan) was used to determine all data. The volume of sample injection was 1 μL.

2.5. LCMS-Q-TOF identification of adipic acid

The purified adipic acid samples and the same amount of adipic acid standard were dissolved in the water. The purified samples were analyzed by Waters MALDI SYNAPT Q-TOF MS equipped with Waters Acquity UPLC BEH C18 column (2.1 mm x 50 mm) and Waters Acquity PDA detector (200–400 nm)(Li et al., 2016). The mobile phase was: A: formic acid (0.1%), B: MeOH (100%) with 0.3 mL min⁻¹ of flow velocity at 45 °C with gradient elution program (Supplementary Table 4). The isocratic elution is MS conditions: ESI⁻, Capillary: 3.5 kV, Cone: 30 V, Source Temp: 100 °C, Desolvation Temp: 400 °C, Desolvation Gas Flow: 700 L h⁻¹, Cone Gas Flow: 50 L h⁻¹, Collision Energy: 6 eV, mass range: 20–2000 m/z, detector voltage: 1800 V.

2.6. NMR identification of adipic acid

Waters 1525 (Milford, MA, USA), a preparative UPLC was used to get the pure adipic acid by picking the peaks of it. Waters 1525 was equipped with Waters XBridge C18 (250 mm L × 10.0 mm i.d.) working at 40 °C with UV–VIS detector (λ = 210 nm). The mobile phase was 80% methanol with isotropic elution. The adipic acid peaks were picked and obtained manually around 10 min of retention time. The adipic acid solution was transferred to a rotary evaporator to get rid of MeOH and then freeze-dried for NMR analysis.

¹H NMR spectra were recorded on a Bruker Avance III (400 MHz) spectrometer (Billérica, MA), using MeOD as a solvent and tetramethylsilane as internal standard. Coupling constants J [Hz] were directly taken from the spectra and were not averaged.

2.7. Metabolite quantification

Cell culture samples (1 mL) were collected by centrifugation at 12,000g for 5 min, and the supernatants were used for detection. Metabolites, such as glucose, glycerol, adipic acid, acetic acid, and butyric acid, were measured by high-performance liquid chromatography (HPLC, Rigol, Suzhou, China) equipped with a HPX-87H ion-exclusion column (Bio-Rad, Hercules, CA, USA) The mobile phase consisted of 5 mM H₂SO₄-acetonitrile (97:3) at a rate of 0.3 mL min⁻¹, and the column temperature was maintained at 30 °C. The organic acids were measured by UV–VIS detector and glucose and glycerol were measured by refractive index (RI) detector.

2.8. Acetyl-CoA detection

Cell culture samples (200 μL; OD (600 nm) = 1) were collected and mixed with 600 μL – 80 °C aqueous 60% (v/v⁻¹) methanol solution to stop all biochemical reactions. Cell pellets were collected by centrifugation at 8000g for 10 min at 4 °C, and cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.4). For cell lysis, 500 μL of 6% perchloric acid (2 mmol L⁻¹ dithiothreitol (DTT)) was added to cells, and following ultrasound fragmentation for 15 min, cellular debris and denatured proteins were removed by centrifugation at 12,000g for 10 min at 4 °C. K₂CO₃ was immediately added to the supernatant, and the pH was adjusted to 3.0 (Takamura and Nomura, 1988). The supernatant was then filtered for HPLC measurement. A C18 chromatography column (ODS2 Hypersil, 5 μm; Thermo Fisher Scientific, Waltham, MA, USA) connected to a UV detector (254 nm) was used for detection using buffer solution A (0.2 mol L⁻¹ sodium phosphate (pH 5)), with buffer solution B (0.25 mol L⁻¹ 800 mL sodium phosphate (pH 5) and 200 mL acetonitrile mixture) used as the mobile phase. The flow velocity was 0.3 mL min⁻¹, and the column temperature was maintained at 25 °C. The gradient elution is shown in Supplementary Table 5.

2.9. Measurements of the intracellular ATP and ADP concentrations

To measure the [ATP]/[ADP] ratio, 0.2 mL of cell culture was mixed with 0.2 mL of 80 °C phenol (equilibrated with 10 mM Tris–1 mM EDTA (pH 8)) supplemented with 0.2-g glass beads (106 m in diameter; Sigma-Aldrich, MO, USA) as previously described (Koebmann et al., 2002b). Treatment and measurement of the intracellular concentrations of ATP and ADP were then performed by using ADP/ATP Ratio Assay Kit (Sigma-Aldrich, MO, USA) as recommended by the manufacturer (Koebmann et al., 2002b).

2.10. Enzyme assays

Cell pellets were collected by centrifugation at 8000g for 10 min at 4 °C, and cells were washed twice with cold phosphate-buffered saline (PBS; pH 7.4). For cell lysis, 500 μL of 6% perchloric acid (2 mmol L⁻¹ dithiothreitol (DTT)) was added to cells, and following ultrasound fragmentation for 5 min, cellular debris were removed by centrifugation at 12,000g 10 min at 4 °C. The UV-2450 spectrometer ( Shimadzu) was used in this study.

For reverse 5-Carboxy-2-pentenoyl-CoA reductase (Tfu 1647) activity: The enzymatic activity derived from pAD3 and pAD4 products and harbored in *E. coli* BL21 (DE3) cells was measured by an increase in absorbance at 340 nm caused by NAD⁻ reduction or at 600 nm by DCIP reduction.

For NAD-dependent activity: The solution consisted of 0.2 mM NAD⁻, 5 mM MgCl₂, 100 μL cell extract, 100 mM Tris–HCl, and 5 mM DTT. The initial reaction needed to add 500 mM adipic acid. The definition of 1 U mg⁻¹ was to obtain 1 μmol NADH min⁻¹ mg⁻¹-protein (Deng et al., 2013).

For FAD-dependent activity: The solution consisted of 100 μM
dichlorophenolindophenol (DCIP), 5 mM MgCl₂, 100 μL cell extract and PBS (pH 7.0) was added to reach 0.9 mL. The initial reaction was activated by adding 0.1 mL 500 mM adipic acid. The definition of 1 U mg⁻¹ was to obtain 1 μmol DCIP min⁻¹ mg⁻¹-protein (Deng et al., 2013). The reductions of DCIP was measured at 600 nm with blue to colorless.

For succinyl-CoA synthetase activity: Quantitative detection of succinyl-CoA synthetase activity was obtained by using a commercial available kit according to manufacturer instructions (Genmed Scientifics, Wilmington, DE, USA). Cells were cleaned with a cleaning solution (reagent A), and after centrifugation at 4 °C for 5 min at 300 g, precipitated and dissolved in lysis buffer (reagent B). After vortexing for 15 s, cells were placed on ice for 30 min. After centrifugation under the same conditions as described previously, the supernatant was used to determine protein concentration. Reagent C (130 μg L⁻¹, Solarbio, Beijing, China) was used to determine protein concentration (mg mL⁻¹).

2.11. SDS-PAGE analysis

SDS-PAGE was performed using the SDS-PAGE gel preparation kit (Solarbio) according to manufacturer instructions. A culture sample (1 mL) was taken 24 h after induction for SDS-PAGE analysis and pelletted by centrifugation at 10,000g. Cell pellets were resuspended in 50 mM Tris–HCl (pH 8.0) and vortexed for 5 min on ice. SDS-containing sample buffers (5 μL) were added to 15 μL of the samples and heated for 10 min, followed by 20 μL of each sample being loaded onto the gel. Gels were electrophoresed at 60 V until the samples fully entered into the concentrate (Schagger, 2006). After 30 min, the voltage was changed to and maintained at 80 V. The gel was fixed for 30 min and stained by Coomassie brilliant blue (Biotechnology company, Shanghai, China) for 3 h and destained twice in 10% acetic acid. Products were visualized using an automatic image-analysis system (Beijing 61 gel-imaging analysis system, WD-9413B, Beijing, China).

3. Results

3.1. Constructing the T. fusca RADP in E. coli

The stoichiometry associated with producing adipic acid from glucose and glycerol based on the RADP is shown in Supplementary Table 6. This indicates that in the presence of 1 mol glucose, 2/3 mol adipic acid would be produced by generating 2 mol adenosine triphosphate (ATP) and 4 mol NADH, resulting in a theoretical yield of 0.54 g adipic acid per g glucose. In the presence of 1 mol glycerol, 1/3 mol adipic acid would be produced by generating 1 mol ATP and 3 mol NADH, with a theoretical yield of 0.52 g-adipic acid per g-glycerol. However, the above theoretical yield calculation was based on the TCA cycle, a branched TCA cycle generating less excess NADH might increase the yield further (Tsuji et al., 2013).

Genes from T. fusca were codon optimized for E. coli and ligated into Duet-1 plasmids, and their expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). E. coli BL21 (DE3) cells harboring pAD1 (Tfu-0875 and Tfu-2399), pAD3 (Tfu-0067 and Tfu-1647), and pAD6 (Tfu-2576 and Tfu-2577) were designated as E. coli Mad136, which produced 0.16 g L⁻¹ adipic acid in M9 medium, with a yield of 0.04 g per g glucose (7.4% theoretical yield) (Fig. 2a). The product was verified by gas chromatography-mass spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis. For NMR analysis, the adipic acid was purified by preparative HPLC, which was then subject to freeze drying after getting rid of methanol. The NMR result showed that the pure product we extracted from the fermentation broth was adipic acid (Supplementary Figure 1) and Nuclear Magnetic Resonance (NMR) analysis.
adipic acid, M9, Lysogeny broth (LB), super optimal broth (SOB), super optimal broth with catabolite repression (SOC), 3-(N-morpholino)propanesulfonic acid broth (MOPS), and terrific broth (TB) media were studied. Among these, SOB produced the highest titer from the Mad136 strain at 0.3 g L\(^{-1}\) adipic acid, with a 0.06 g per g glucose yield (an 11.1% theoretical yield) (Fig. 2a).

The Mad136 strain was then cultured in a 5-L bioreactor with 0, 1, 2 and 3 vvm aeration and 400-rpm agitation in SOB medium (Supplementary Figure 3). There was very low adipic acid accumulation in the absence of aeration. However, once the aeration was increased to 1 vvm, the yield was increased to 18.5% theoretical yield. However, we increased the aeration to 2 vvm and 3 vvm, the yield of adipic acid decreased slightly, indicating that high levels of aeration were unfavorable for adipic acid synthesis.

### 3.2. Optimization of the adipic acid pathway

To determine the optimal combination of heterologous enzymes catalyzing the reactions necessary for adipic acid synthesis in *E. coli*, enzyme activities were studied in *E. coli* BL21 (DE3) cells grown in M9 medium to eliminate any trace carbon other than glucose. The production of adipic acid varied significantly along with different combinations of enzymes (Fig. 3). BL21 (DE3) cells harboring pRSF-Tfu\_0875-Tfu\_2399, pET-Tfu\_0067-Tfu\_1647, pCD-Tfu\_2576-Tfu\_2577 and pRSF-PaaJ-PaaH1, pET-Tfu\_0067-Tfu\_1647, pCD-Tfu\_2576-Tfu\_2577 produced the highest adipic acid yield (0.2 g L\(^{-1}\)), whereas those harboring pRSF-Tfu\_0875-Tfu\_2399, pET-Ech-Ter, pCD-Buk1-Pb, pRSF-PaaJ-PaaH1, pET-Ech-Ter, and pCD-Buk1-Pb accumulated the lowest adipic acid yield (0.06 g L\(^{-1}\)) from 4 g L\(^{-1}\) glucose. These results demonstrated that strains expressing Ech and Ter genes produced significantly less adipic acid than those harboring Tfu\_0067 and Tfu\_1647. Our results indicated that the presence of the Ter gene was homologous to Tfu\_1647 in regard to adipic acid production. Deng et al. (Deng and Mao, 2015) reported that Tfu\_1647 was the rate-limiting step for adipic acid synthesis in *T. fusca*. Therefore, we hypothesized that low levels of Tfu\_1647 might hinder adipic acid accumulation. To test this hypothesis, an artificial operon including sequences for Tfu\_0067 and Tfu\_1647 was synthesized and ligated into pTrc99A, a medium copy number plasmid. Based on the enzymatic assay, Tfu\_1647 was confirmed to be the rate-limiting step for adipic acid synthesis in *E. coli*.

**Fig. 3.** Combinations of heterologous enzymes catalyzing the reactions necessary for adipic acid synthesis. PaaJ: β-ketoacyl-CoA thiolase, PaaH1: 3-hydroxyacyl-CoA reductase; Ech: enoyl-CoA hydratase; Ter: trans-enoyl-CoA reductase; Buk1: butyryl kinase; Pb: phosphate butyryltransferase. Tfu\_0875: β-ketothiolase, Tfu\_2399: 3-hydroxyacyl-CoA dehydrogenase, Tfu\_0067: 3-hydroxyadipyl-CoA dehydrogenase, Tfu\_1647: 5-Carboxy-2-pentenoyl-CoA reductase, and Tfu\_2576-7: adipyl-CoA synthetase. Error bars represent the s.d. from three independent assays. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 4.** Over-expression of Tfu\_1647 by pTrc99A. (a) SDS-PAGE results. Lane 1, marker; lanes 2-6: protein lysates isolated from strains BL21 (wild-type; lane 2) and BL21 (pET-Tfu\_0067-Tfu\_1647; lane 3), BL21 (pET-Tfu\_0067-Tfu\_1647; lane 4), BL21 (pRSF-Tfu\_0875-Tfu\_2399, pET-Tfu\_0067-Tfu\_1647, and pCD-Tfu\_2576-Tfu\_2577; lane 5), and BL21 (pRSF-Tfu\_0875-Tfu\_2399, pET-Tfu\_0067-Tfu\_1647, and pCD-Tfu\_2576-Tfu\_2577; lane 6). Strains were grown in SOB medium containing 0.4% glucose and induced with 1 mM IPTG. Samples were separated by SDS-PAGE. Tfu\_1647 bands at 42.2 kDa are identified by black arrow and the suspected Tfu\_0067 bands at 28.2 kDa are identified by blue arrow. Full gel for the portion of gel presented here was included in Supplementary Figure 10. (b) Specific reverse Tfu\_1647 activity. Error bars represent the s.d. from three independent assays. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pET-Tfu\_1647 (Supplementary Table 7), indicating that the translation of Tfu\_1647 on pTrc-Tfu\_1647 might be much stronger than that of pET-Tfu\_1647. Based on the enzymatic assay, Tfu\_1647 was confirmed to be
FADH2-dependent. Bioinformatics analysis indicates this enzyme belongs to the Bcd-type family of enoyl-CoA reductases, which are reversible (Deng and Mao, 2015). We measured the reverse activity of Tfu_1647: converting from adipyl-CoA to 2,3-dehydroxy adipyl-CoA. The reverse activities of Tfu_1647 following Tfu_1647 expression controlled by the pTrc promoter and T7 promoter were shown in Fig. 4b. The specific reverse Tfu_1647 activity observed after 24, 28 and 44 h of fermentation was 0.72 U mg\(^{-1}\), 0.81 U mg\(^{-1}\), and 1.48 U mg\(^{-1}\), respectively, for the T7 promoter and 3.1 U mg\(^{-1}\), 1.83 U mg\(^{-1}\), and 2.21 U mg\(^{-1}\), respectively, for the pTrc promoter. Thus, the expression of Tfu_1647 controlled by pTrc promoter was much stronger than the one controlled by T7 promoter and it was also confirmed by the RBS results above.

The final step of the RAPD was catalyzed by Tfu_2576-7 subsequently generating ATP as the product. Therefore, we measured ATP levels in E. coli strains with and without the ability to produce adipic acid (Fig. 2b). Our results showed that during the log phase, ATP/ADP ratios in strain Mad146 were 7.53, which was 4.8-fold greater than that observed in BL21 (DE3) cells (1.57). During the stationary phase, the ATP level measured in the Mad146 strain was 4.50, which was 4.4-fold greater than that observed in BL21 (DE3) cells (1.02). These results indicated that overexpression of the heterologous enzymes allowed Mad146 cells to accumulate significantly more ATP as compared with BL21 (DE3) cells.

The new strain (Mad146) containing pAD1, pAD4, and pAD6 was grown in SOB medium in the presence of 4 g L\(^{-1}\) glucose with 49.5% theoretical yield in shake flasks. (Fig. 5a), which was 4.5-fold higher than that observed from strain Mad136. These results confirmed that the production of adipic acid was correlated with Tfu_1647 expression level.

3.3. Engineering the host strain

We deleted L-lactate dehydrogenase (ldhA), succinyl-CoA synthetase alpha subunit (sucD), and acetyl-CoA acetyltransferase (atoB) individually to test the effects of their knock out on adipic acid synthesis. We employed CRISPR/Cas9 (Sander and Joung, 2014) to remove ldhA in E. coli and observed no lactic acid accumulation in the ldhA-deletion strain, and thus adipic acid yield in the new strain (Mad1146) was increased from 49.5% to 61.7% theoretical yield in shaken flasks (Fig. 5a).

Butyric acid is synthesized from acetoacetyl-CoA and catalyzed by enzymes encoded by Hbd, Crt, Ter, and tesB (Saini et al., 2014; Yu et al., 2015), which are homologous to the Tfu_2399, Tfu_0067, Tfu_1647, and Tfu_2576-7, respectively, used in this study. The fermentation analysis also showed that butyric acid was another byproduct. To eliminate butyric acid, we deleted atoB encoding acetyl-CoA acetyltransferase involved in the conversion of acetyl-CoA to acetoacetyl-CoA in E. coli. The fermentation results using strain Mad2146 revealed no butyric acid produced and an increase in adipic acid from a 49.5–68.5% theoretical yield in shaken flasks (Fig. 5a).

To switch the carbon flux of succinyl-CoA from succinic acid to adipic acid, sucD encoding succinate-CoA ligase subunit alpha was deleted in E. coli to test whether partial inhibition of succinate production from succinyl-CoA would direct carbon flux from succinyl-CoA to adipic acid through the heterologous pathway. The specific succinyl-CoA synthetase activity of the ΔsucD strain Mad3146 was 0.0068 μmol min\(^{-1}\), which was ~50% of that of the parent strain Mad146 (0.0103 μmol min\(^{-1}\)), indicating that the degradation of succinyl-CoA to succinate was largely blocked. The adipic acid yield of strain Mad3146 (a 67.6% theoretical yield) increased significantly as a result compared with that observed in strain Mad146 (a 49.5% theoretical yield) in shaken flasks.

Based on these results, we deleted the sucD, atoB, and ldhA genes in BL21 (DE3) cells harboring plasmids for adipic acid production, resulting in strain Mad123146. Our results revealed no byproducts other than adipic acid produced by this strain (Fig. 5a), whereas adipic acid production reached a 93.1% theoretical yield in shaken flasks, which was 6.7-fold higher than that observed from strain Mad136.

In order to test the stability of the recombinant E. coli strain for industrial application purpose, the strain Mad123146 was grown on 4 g L\(^{-1}\) glucose in shake flasks and the production of adipic acid was induced by IPTG. Once the cells reached stationary phase, cells were harvested and transferred to the fresh medium with IPTG in it to start producing adipic acid. In this study, we transferred cells for 10 times. In Supplementary Figure 4, decreased production of adipic acid from glucose was not observed throughout these transfers, confirming the stability of the recombinant E. coli strain.

Given that acetyl-CoA represents another adipic acid precursor, we further compared the intracellular concentration of acetyl-CoA in the parent and engineered strains. Acetyl-CoA levels in different strains are shown in Fig. 5b. Strains Mad1, Mad2, Mad3, and Mad23 exhibited 1.2-fold higher acetyl-CoA concentrations relative to the BL21 (DE3) strain, whereas strain Mad123 (ΔldhA, ΔatoB, and ΔsucD) exhibited 1.6-fold higher acetyl-CoA concentrations as compared with the BL21 (DE3) strain. These results indicated that higher acetyl-CoA levels were beneficial for adipic acid production in the engineered E. coli strain.
3.4. Production of adipic acid in 5-L bioreactors

To achieve high titers of adipic acid, fed-batch fermentation was employed using glycerol as the major substrate. Because the adipic acid production was sensitive to oxygen, we conducted fed-batch fermentation by not controlling oxygen (1 vvm aeration, 400 rpm agitation), and by controlling dissolved oxygen (DO) at 5% and 10%, respectively. In Supplementary Figure 5, although higher levels of DO helped accumulate additional biomass as compared with that accumulated at low levels of DO, the maximal titer of adipic acid production was observed at low oxygen levels (68.0 g L\(^{-1}\), 42.3 g L\(^{-1}\) and 36.1 g L\(^{-1}\) for not control, DO 5% and DO 10%, respectively). The results of adipic acid production in the absence of DO control are shown in Fig. 6. We observed a maximal adipic acid titer of 68.0 g L\(^{-1}\) after an 88 h fermentation, with a maximal OD (600 nm) of 36.7 achieved after 64 h, representing a 226 fold increase as compared with strain Mad136. The DO under this condition was < 5% for most of the fermentation process.

The fermentation broth was then subject to the purification process to extract adipic acid (the general process is shown in Supplementary Methods). After purification, adipic acid was subjected to the LCMS-Q-TOF analysis with gradient elution (Supplementary Table 4). The purity of adipic acid was 96.86% with a yield of 87.3%. The above data are calculated according to the peak area according to the Supplementary Figure 6.

4. Discussion

Adipic acid is a dicarboxylic acid that plays an important role in chemical industry, medicine, and lubricant manufacturing, with the most popular product derived from adipic acid being nylon-6, 6 (Deng et al., 2016; Polen et al., 2013; Vardon et al., 2015; Yu et al., 2014). Previously, no native adipic acid synthesis pathway was found in microorganisms until the discovery of a RADP by Deng et al. in the actinobacterium T. fusca capable of converting acetyl-CoA and succinyl-CoA to adipic acid (Deng and Mao, 2015). Here, we constructed the T. fusca RADP in E. coli BL21 (DE3) by expression of five enzymes (Tfu_0875, Tfu_2399, Tfu_0067, Tfu_1647, and Tfu_2576-7) for adipic acid production via Duet-1 plasmids. Our results indicated that the engineered E. coli strain accumulated only small amounts of adipic acid. Subsequent analysis revealed that low expression of Tfu_1647 constituted the rate-limiting enzyme involved in decreased adipic acid accumulation, with overexpression of Tfu_1647 by pTrc99A resulting in high yields of adipic acid.

The strategies used to increase adipic acid yield were as follows: 1) partial inhibition of succinate production from succinyl-CoA to increase the accumulation of succinyl-CoA, the precursor of adipic acid; 2) deletion of atoB to eliminate butyric acid production; and 3) deletion of ldhA to eliminate lactic acid production. Due to the low efficiency of homologous recombination in E.coli BL21 (DE3) aided by the λ-Red system, the CRISPR/Cas9 system was employed in this study to enable gene deletion (Jiang et al., 2015).

The bioinformatics analysis indicated that Tfu_1647 belonged to the Bcd-type family of enoyl-CoA reductases (Deng and Mao, 2015), which was confirmed by enzymatic assay. Thus, this enzyme was reversible, with its directionality dependent upon relative concentration and redox state. Highly aerobic conditions promote adipate degradation, given that adipic acid production was reduced at high aeration rates. Higher aeration rates enhanced respiration, which competed for the reduced potential for producing adipic acid and increased cellular yield unassociated with adipic acid production. However, in the complete absence of oxygen, the TCA cycle would be too weak to generate sufficient succinyl-CoA for adipic acid production.

During the late stationary phase, acetate might be assimilated by cells. Gupta et al. found the same phenomenon and they observed that the acetate could be reassimilated (Gupta et al., 2017). According to the stoichiometry of acetate production, acetate is generated from acetyl-CoA by phosphate acetyltransferase and acetate kinase while generating ATP (Kim et al., 2015; Kumari et al., 2000). However, this reaction is reversible and converted acetate to acetyl-CoA. The reverse acetate-synthesis reaction might also provide higher levels of acetyl-CoA, which is the key precursor for adipic acid synthesis. Another explanation involves the acetyl-coenzyme A synthetase gene (acs) (Webster, 1966; Webster, 1967), which is silent during the early stages of growth and can be activated upon acetate accumulation in the broth during the late stationary phase in response to rising cyclic adenosine monophosphate (cAMP) levels, low oxygen levels, and carbon flux through pathways associated with acetate metabolism (Kumari et al., 2000).

Succinyl-CoA synthetase catalyzes the conversion of succinyl-CoA to succinate by generating ATP (Litsanov et al., 2012). However, succinyl-CoA is a precursor for adipic acid synthesis. A reasonable pool of succinyl-CoA necessary to drive carbon flux toward adipic acid synthesis is highly important. Here, rather than deleting all genes encoding succinyl-CoA synthetase, the gene sucD encoding the alpha subunit was deleted in E. coli BL21 (DE3), resulting in the downregulation of succinate synthesis (Cheong et al., 2016). We found that sucD deletion significantly increased adipic acid production. Although Tfu_2576-7 is annotated as a succinyl-CoA synthetase, the similarity between
Table 1
Comparison of cell factories for production of adipic acid.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Media</th>
<th>Cultivation mode</th>
<th>Titer (g L⁻¹)</th>
<th>productivity (g/l/h)</th>
<th>Yield (g/g)</th>
<th>Yield (% theoretical yield)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>R/2</td>
<td>Shake flask</td>
<td>0.000673</td>
<td>0.0000056</td>
<td>0.0000673</td>
<td>0.012</td>
<td>(Yu et al., 2014)</td>
</tr>
<tr>
<td>E. coli</td>
<td>MOPS</td>
<td>Batch</td>
<td>2.5</td>
<td>0.017</td>
<td>0.05</td>
<td>9.47</td>
<td>(Cheong et al., 2016)</td>
</tr>
<tr>
<td>E. coli</td>
<td>N.A.</td>
<td>Shake flask</td>
<td>2.03652</td>
<td>N.A.</td>
<td>0.202</td>
<td>37.41</td>
<td>(Draths and Frost, 1994)</td>
</tr>
<tr>
<td>Thermolysina fusca B6</td>
<td>Hagedahl</td>
<td>Batch</td>
<td>2.23</td>
<td>0.034</td>
<td>0.0446</td>
<td>8.26</td>
<td>(Deng and Mao, 2015)</td>
</tr>
<tr>
<td>E. coli</td>
<td>SOB</td>
<td>Fed-batch</td>
<td>68.0</td>
<td>0.810</td>
<td>0.378</td>
<td>72.7</td>
<td>This study</td>
</tr>
</tbody>
</table>

*N.A.: not available.

Tfu_2576-7 and sucC-D is ≈47% (Supplementary Figure 7); therefore, the absence of sucD could not be fully compensated by Tfu_2577 in strain Mad123146 (AsucD).

For NADH generation, it is obvious that the strain Mad123146, which was deficient in both lactate dehydrogenase (encoded by ldhA) and acetyl-CoA acetyltransferase (encoded by atob), showed significantly higher NADH concentration than that of Mad136, throughout the whole fermentation process (Supplementary Figure 8). The elevated NADH could significantly drive the conversion of succinyl-CoA and acetyl-CoA to 3-hydroxyadipyl-CoA, which is the second step of the reverse adipate-degradation pathway (RADP).

The cells from mid-log phase during batch culture process were harvested and tested for the intracellular succinyl-CoA concentrations (Bennett et al., 2009). The results are shown in Supplementary Figure 9. The engineered strains without sucD (Mad3146, Mad23146 and Mad123146), a subunit of succinate-CoA ligase, showed much higher intracellular succinyl-CoA concentrations than those strains with sucD (Mad146 and Mad1146). Besides, the final strain Mad123146 had the highest level of succinyl-CoA (0.0168 mmol OD (600 nm). Thus, succinyl-CoA, the precursor of adipic acid was accumulated in engineered strains to drive the production of adipic acid.

During the aerobic fermentation, glycerol is transported to E. coli by a glycerol transporter (gplF), which is then converted to Glycerol-3-phosphate by a glycerol kinase (glpK). Two respiratory glycerol-3-phosphate dehydrogenases (G3PDHs, encoded by the glpD and glpABC) then convert sn-Glycerol-3-phosphate to Glycerine phosphate (dihydroxyacetone Phosphate, DHAP), which enters glycolysis pathway (Murarka et al., 2008).

Koebmann et al. showed that in the aerobic condition, at least 75% of the control over glycolysis of E. coli related to the in the ATP-consuming reactions (Koebmann et al., 2002b). Based on above results, they over-expressed the genes encoding part of the F1 unit of the (F1F0) H⁺-ATPase, resulting in a 70% increase in the glycolytic flux. The same strategy was successful to uncouple of glycolysis from biomass production in Lactococcus lactis (Koebmann et al., 2002a). Thus, the next step, we might introduce the ATP hydrolysis by over-expressing F1 unit of the (F1F0) H⁺-ATPase in E. coli and channel more flux to the target product, adipic acid.

5. Conclusions

In this study, we optimized the adipic acid-synthesis pathway from T. fusca in E. coli BL21 (DE3) by rational deletion of genes hindering adipic acid production, resulting in a maximal adipic acid titer of 68.0 g L⁻¹ from glycerol as the major substrate. To the best of our knowledge, this constitutes the highest titer reported in E. coli (Table 1).

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Conflicts of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2018.04.002.

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Koebmann, B.J., Solem, C., Pedersen, M.B., Nilsson, D., Jensen, P.R., 2002. Expression of genes encoding F1-ATPase results in uncoupling of glycolysis from biomass production in Lactococcus lactis(Koebmann et al., 2002a). Thus, the next step, we might introduce the ATP hydrolysis by over-expressing F1 unit of the (F1F0) H⁺-ATPase in E. coli and channel more flux to the target product, adipic acid.

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