Metabolic Engineering 51 (2019) 121–130

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Efficient production of 3-hydroxypropionate from fatty acids feedstock in *Escherichia coli*

Bo Liu[a,b], Shuman Xiang[a,b], Guang Zhao[c], Bojun Wang[a], Yanhe Ma[a,*], Weifeng Liu[a,*], Yong Tao[a,b]

[a] CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China

ARTICLE INFO

Keywords:
Fatty acids
Biomass resource
3-hydroxypropionate
Metabolic engineering
Atomic economy
Escherichia coli

ABSTRACT

The production of chemicals from renewable biomass resources is usually limited by factors including high-cost processes and low efficiency of biosynthetic pathways. Fatty acids (FAs) are an ideal alternative biomass. Their advantages include high-efficiently producing acetyl-CoA and reducing power, coupling chemical production with CO_2 fixation, and the fact that they are readily obtained from inexpensive feedstocks. The important platform chemical 3-hydroxypropionate (3HP) can be produced from FAs as the feedstock with a theoretical yield of 2.49 g/g, much higher than the theoretical yield from other feedstocks. In this study, we first systematically analyzed the limiting factors in FA-utilization pathways in *Escherichia coli*. Then, we optimized FA utilization in *Escherichia coli* by using a combination of metabolic engineering and optimization of fermentation conditions. The 3HP biosynthesis module was introduced into a FA-utilizing strain, and the flux balance was finely optimized to maximize 3HP production. The resulting strain was able to produce 3HP from FAs with a yield of 1.56 g/g, and was able to produce 3HP to a concentration of 52 g/L from FAs in a 5-L fermentation process. The strain also could produce 3HP from various type of FAs feedstock including gutter oil. This is the first report of a technique for the efficient production of the platform chemical 3HP from FAs.

1. Introduction

The production of industrial and pharmaceutical chemical products from renewable biomass resources is a promising technique to replace conventional fossil-based manufacture, which has serious problems such as finite resources, high CO_2 emissions, and environmental pollution (Hernandez et al., 2014; Kircher, 2015; Stephanopoulos, 2007). However, biomass routes are often unable to compete with petrochemical routes at the industrial level (Chubukov et al., 2016; Gustavsson and Lee, 2016; Sanford et al., 2016). This is mainly due to the high cost of production from biomass, compared with the lower cost of production from petrochemical resources (Kircher, 2015; Sanford et al., 2016). There are several reasons for it. For example, the shortage of agricultural land and the increasing food demand might lead to higher crop-based feedstock prices (Kluts et al., 2017). Besides, the targeted chemical biosynthesis pathways with low atomic economy will result in reduction of theoretical production yield, titer, and productivity, making the process inefficient. (Chae et al., 2017; Gustavsson and Lee, 2016).

To overcome these issues, a number of studies have been conducted to search for new biomass sources with more efficient processes. Several non-food biomasses, including lignocellulose, protein waste, have been developed and extensively studied (Choi et al., 2014; Kawaguchi et al., 2016; Wheeldon et al., 2017). Recently, utilizing CO_2 as carbon resource for the production of chemicals has attracted more attention, due to the fact that not only is CO_2 a more sustainable resource, but also the utilization of CO_2 can be helpful to reduce CO_2 accumulation (Antonovsky et al., 2016). Yet all of these feedstock systems have their

Abbreviations: 3HP, 3-hydroxypropionate; FAs, fatty acid(s); PFAD, palm fatty acid distillate; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced form); $NADP^+$/NADPH, nicotinamide adenine dinucleotide phosphate (oxidized/reduced form); $FADH^+$/$FADH_2$, flavin adenine dinucleotide (oxidized/reduced form); LB, Luria-Bertani medium; OD_{600}, optical density (measured at a wavelength of 600 nm); DO, dissolved oxygen concentration; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HESI, heated electrospray ionization; HFIP, hexafluoro-2-propanol; TEA, triethylamine; SRM, selected reaction monitoring; FWHM, full width at half maximum; GC, gas chromatograph; FAMEs, fatty acid methyl esters; CCM, carbon-concentrating mechanism; ACC, acetyl-CoA carboxyltransferase complex; BT, bicarbonate transporter; CA, bicarbonate transporter

* Corresponding authors.
E-mail addresses: mayanhe@im.ac.cn (Y. Ma), liuwfv@im.ac.cn (W. Liu).

https://doi.org/10.1016/j.ymben.2018.10.003
Received 15 August 2018; Received in revised form 10 October 2018; Accepted 14 October 2018
Available online 19 October 2018

own drawbacks. Utilization of carbohydrate biomass (e.g. lignocelluloses) is usually associated with loss of carbon, making the biosynthesis pathway inefficient (Wheeldon et al., 2017). When use CO_2 as resource, biological CO_2-fixing reactions always need energy or strictly catalytic conditions (Hu et al., 2018). Therefore, a more efficient biomass utilization strategy is required.

Here, we propose that fatty acids (FAs) are not only an ideal alternative biomass with the dual advantages of high atomic economy and low cost, but they also can drive fixation of CO_2 or other inorganic carbon compounds, consequently further improving carbon utilization efficiency. FAs, which have highly reduced long aliphatic hydrocarbon chains, are primarily catabolized through the β-oxidation pathway, resulting in the formation of the key intermediate, acetyl-CoA (Kim and Battaile, 2002). The formation of acetyl-CoA is very efficient, as it results in 100% carbon recovery. Acetyl-CoA is a common precursor for diverse CO_2 fixation pathways (e.g., the Fuchs-Holo bicycle and the hydroxypropionate/hydroxybutyrate (HP/HB) cycle) (Erb, 2011), thus it can mediate the assimilation of CO_2 into target chemicals. Meanwhile, the β-oxidation of FAs generates a large amount of reducing power, which can be used for carbon fixation and further biosynthetic pathways. Therefore, utilization of FAs as feedstock provides a more green and economical route for chemical production with advantages in both energy and precursor supply.

Fatty acids can be obtained from various cheap feedstocks. For example, palm FA distillate (PFAD), a by-product of palm oil production, contains about 65.70–94.68% free FAs (Chang et al., 2016). A rough estimate of the global annual supply of PFAD is 2.98 million tons, and it is considered to be a potential alternative low-cost non-food feedstock (ZERO, 2016). The international price of PFAD in 2018 is about 350–600 USD/ton, which is close to the price of glucose (Alibaba.com, URL: https://www.alibaba.com/trade/search?fsb=y&IndexArea=product_en&CatId=&SearchText=PFAD). Waste cooking oil (gutter oil) is another cheap source of FAs. More than 13.74 million tons of gutter oil were produced in China in 2010 (Zollinger, 2015). A technique has been developed to convert gutter oil into biodiesel (Liang et al., 2013). Thus, the development of a biomanufacturing route from FAs will also help to solve the problems involved in waste and by-product utilization.

Thus, we use FAs as feedstock to produce 3-hydroxypropionate (3HP), an important precursor for the synthesis of bioplastics. 3HP has been listed as one of the 12 biology-based chemical products with the most potential by the Department of Energy, USA (Werpy and Peterson, 2004). Many important chemical products such as acrylic acid, methacrylic acid, propylene glycol, and acrylamide can be synthesized from 3HP (de Fouchecour et al., 2018; Liu et al., 2017). Several 3HP biosynthetic pathways have been reported: it can be biosynthesized from glucose through either β-alanine or malonyl-CoA routes (Borodina et al., 2015; Kildegaard et al., 2016; Liu et al., 2016, 2018a; Song et al., 2016), or it can produced using glycerol or other C3 chemicals as the precursor (Kim et al., 2014; Luo et al., 2016; Seok et al., 2018; Zhao et al., 2015). The production of 3HP from FAs as a biomass resource represents a novel route with significant advantages. First, FAs can efficiently supply acetyl-CoA as the precursor for 3HP biosynthesis without the loss of carbon (Fig. 1). Acetyl-CoA is then converted into malonyl-CoA by a CO_2/carbonate fixation reaction. ATP needed for carbon fixation is ultimately supplied from β-oxidation. This further reduces the consumption of the FAs source (Fig. 1). The biosynthesis of 3HP through this pathway requires 2 moles of NAPDH, which can be exactly provided by the excess reducing power generated by β-oxidation (Fig. 1). The theoretical yield of 3HP production from the 16-carbon FA palmitic acid is 2.49 g/g, much higher than its theoretical yield from other feedstocks (Fig. 1, Table 1).

The microbial production of chemicals from FAs is still limited by several factors. Most microbial strains cannot efficiently utilize FAs (Dellomonaco et al., 2010). The metabolic pathways of FA utilization in the most common industrial host, *E. coli*, must be systematically engineered to utilize FAs efficiently (Dellomonaco et al., 2010). Furthermore, when cells use FAs as the carbon source, the global metabolic network differs substantially from that when glucose is used as the carbon source (Feng and Cronan, 2012). Therefore, it is important to determine how to balance FAs utilization, the biosynthesis of target chemicals, cell growth and metabolism. To achieve this, we analyzed the FAs metabolic pathways in *E. coli*, and then conducted systematic metabolic engineering to improve the uptake and utilization of FAs. The 3HP biosynthesis module was introduced into a FAs-utilizing strain of *E. coli*, and the flux balance was finely optimized to maximize 3HP production. Our study has, for the first time, elucidated a potential route for the efficient production of the platform chemical 3HP from FAs.

2. Materials and methods

2.1. Strains and agents

The *E. coli* strains DH5α and BW25113/F′ were used as host strains for plasmid construction and 3HP biosynthesis, respectively. The 3HP solution (30%) was purchased from TCI (Tokyo, Japan). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) or were local products of analytical grade. Fast Pfu Fly™ DNA polymerase was obtained from TransGen Biotech Co., Ltd (Beijing, China). A Gibson Assembly® Cloning Kit (New England Biolabs, Beverly, MA, USA) was used for plasmid construction. All strains and plasmids used are listed in Table 2. Primers sequences are provided in Supplementary Table S1. Detailed plasmids information are provided in Supplementary Table S2.

Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used to grow *E. coli* cells unless otherwise specified. Auto-induction medium (Studier, 2005), which is based on diauxic growth in mixtures of glucose and inducer arabinose, was used to induce the expression of pathway proteins to minimize user intervention before bioconversion. Where necessary, antibiotics were added (to a final concentration of 100 mg/L for carbenicillin, 50 mg/L for kanamycin, 50 mg/L for streptomycin, and 34 mg/L for chloramphenicol). The whole-cell bioconversion for FAs utilization/3HP production was carried out using M9 FAs medium containing 12.8 g/L $Na_2HPO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, and 10 g/L palmitic acid (emulsified with 2 g/L Brij 58). The M9 FAs medium was supplemented with 40 mg/L biotin and 20 mM $NaHCO_3$ for 3HP production, and with 0.1 mM $CaCl_2{\cdot}2H_2O$ and 2 mM $MgSO_4{\cdot}7H_2O$ for the FAs utilization study. For the nitrogen source study, NH_4Cl was replaced by various amino acids or yeast extract at given concentrations. For other FA-sources study, 10 g/L palmitic acid was replaced by 40 g/L soybean oil or 40 g/L gutter oil (From local manufacturing plant), and 15 mg/L lipase was added intermittently.

2.2. DNA manipulation and genome editing

Molecular cloning was performed using standard protocols (Green and Sambrook, 2012). Gene-deletion strains were obtained from the Keio Collection (Baba et al., 2006). For genome editing, including chromosomal promoter replacement and gene integration, the fragments of CPA1 promoter, 119 promoter, tac promoter, and P119-*bt-ca* cassette, each flanked with Lox66-kan^r-Lox71 cassette, were amplied from pSLC1k, pSL91k, pSLT1k, and pSL91k-BC, respectively. Then, the gene-Lox66-kanr-Lox71 fragments were assembled with about 500 nt of the upstream/down region of target genes by overlap-extension PCR, resulting in the target fragments. Promoter replacement and gene integration were carried out using λ Red recombineering, as previously described (Datsenko and Wanner, 2000).

Mutant strains were verified by PCR and further confirmed by sequencing (GENEWIZ, Suzhou, China). The kanamycin resistance marker was eliminated using the plasmid pS195s-Cre by adding 0.2% L-arabinose or by using pCP20. For chromosomal integration of multiple

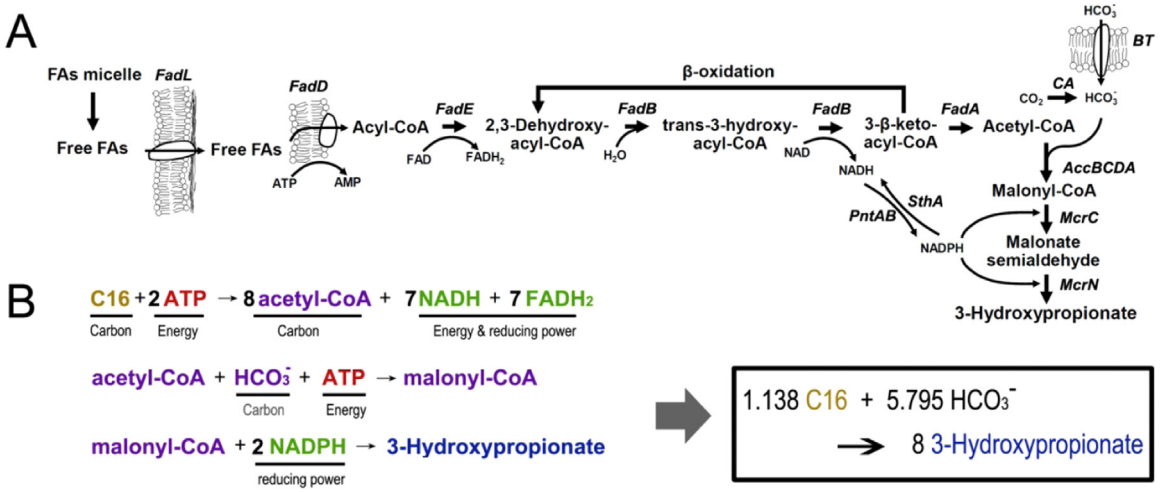


Fig. 1. Production route of 3-hydroxypropionate (3-HP) from fatty acids (FAs). A. Enzymes involved in 3HP biosynthesis pathway. FadL, long-chain fatty acid outer membrane porin; FadD, fatty acyl-CoA synthetase; FadK, short chain acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadB, α component of the fatty acid oxidation complex; FadJ, α component of the anaerobic fatty acid oxidation complex; FadA, β component of the fatty acid oxidation complex; FadI, β component of the anaerobic fatty acid oxidation complex; AccBCDA, acetyl-CoA carboxyltransferase complex; McrC, malonyl-CoA reductase C-domian; McrN, malonyl-CoA reductase N-domain; SthA, soluble pyridine nucleotide transhydrogenase; PntAB, pyridine nucleotide transhydrogenase; BT, bicarbonate transporter; CA, bicarbonate transporter. B. Equations from palmitic acid to 3HP. C16, palmitic acid; equation in box shows overall theoretical stoichiometry.

Table 1
Theoretical yield of 3-hydroxypropionate production from different feedstocks.

Carbon source	Pathway	Theoretical yield g/g
Glucose	Malonyl-CoA pathway	1.00
Glucose	β-Alanine pathway	1.00
Glycerol	CoA-dependent/ independent pathway	0.98
Palmitic acid (16-carbon fatty acid)	Malonyl-CoA pathway	2.49

targets, P1 virus-mediated transfection was performed, as described elsewhere (Thomason et al., 2007).

2.3. Determination of cell growth

Cell growth could not be monitored by measuring changes in OD_{600} because FA droplets made the medium opaque. Therefore, we developed an assay based on the intensity of green fluorescent protein (GFP). The *GFP* gene was placed under the control of the P119 promoter to provide relatively constant expression levels in different media and at different growth stages (see Supplementary Fig. S3). This allowed us to monitor the intensity of GFP to reflect the change in biomass during cell growth.

To evaluate cell growth in FAs-medium, *E. coli* strains harboring the GFP expression plasmid pS95s-GFP were cultured overnight in LB medium, washed with 0.85% NaCl, suspended in 50 μL (OD_{600} = 5) 0.85% NaCl solution, and subinoculated (1:100) into 5 mL M9 salt FAs medium (37 °C, 220 rpm). A 200-μL aliquot of the culture was sampled every 75 min and frozen at − 20 °C, and then the fluorescence intensity was measured using a BioTek Synergy Mx microplate reader (BioTek, Winooski, VT, USA) in a single plate. The following filter settings were applied: excitation at 468 nm (band width, 20 nm) and emission at 512 nm (bandwidth, 20 nm). Fluorescence intensity is given in relative fluorescence units. All data were obtained from three replicates.

2.4. Transcriptomic analysis

Strain FA01 was cultured in M9 salt FAs medium or M9 salt glucose medium (where palmitic acid in the M9 salt FAs medium was replaced with 1% glucose) in 500-mL flasks. The growth curves in both M9 salt FAs medium and M9 salt glucose medium were first recorded using GFP intensity measurement to determine the exponential growth phase. Then the strains were cultured under the same conditions to mid-exponential growth phase. All strains were cultured with three replicates. The cells were harvested by centrifugation and washed three times with 0.85% NaCl solution, and then frozen on dry ice immediately. RNA extraction and purification, transcriptome enrichment, next-generation sequencing, and transcriptomic data analysis were carried out by GENEWIZ Inc. (Suzhou, China).

2.5. Whole-cell bioconversion for FAs utilization and 3HP production

For the FAs-utilization study, strains were grown at 37 °C in LB medium to the post-exponential growth phase. Cells were collected by centrifugation at 5000 × *g* for 10 min, washed twice with 0.85% NaCl solution, and then transferred into M9 FAs medium at a starting OD_{600} of either 10 or 30 in pre-experiments and starting OD_{600} of 30 in final experiments. Bioconversion was performed in tubes at 37 °C with shaking at 220 rpm for 2–5 h. Then, free FAs were measured, as described in Section 2.7. For *de novo* biosynthesis of 3HP from FAs, strains were grown in LB medium overnight, then transferred into auto-induction medium and cultured for 12 h (37 °C, 220 rpm) to induce the expression of pathway genes. Bioconversion reactions were performed in tubes at 37 °C at a starting OD_{600} of 30 with shaking at 220 rpm for 20 h. The 3HP concentration was then determined by HPLC as described in Section 2.7. All data were obtained from three replicates.

2.6. Batch and fed-batch fermentations

Fermentations were carried out in a fermenter (Bailun Bio, Shanghai, China) containing 2 L minimal medium (10 g/L glucose, 14 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, and 1.8 g/L citric acid monohydrate). The pH was controlled at 7.0 via automated addition of ammonia, and the dissolved oxygen (DO) concentration was controlled by adjusting the agitation speed and airflow rate during fermentation. For seed preparation, strains were cultivated on LB agar plates using plate streaking at 37 °C over night and monoclonal colony was inoculated to 5 mL LB medium. After overnight cultivation, the strain was then inoculated to 100 mL LB medium, and was transfered to 5 L fermenter when OD_{600} reached 2. After the initial glucose was nearly exhausted, 2 g/L L-arabinose was added when the OD_{600} reached 15 to induce the

Table 2
Strains and plasmids used in this study.

Strain	Genotype	Source
E. coli BW25113/F′	*rrnBT*14 Δ*lacZWJ*16 *hsdR*514 Δ*araBADAH*33 Δ*rhaBADLD*78 [F′ *proAB lacIqZ*ΔM15 *Tn*10 (*Tetr*)]	CGSC[a]
FA01	*E. coli* BW25113/F′, Δ*fadR*	This study
FA02	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*atoSC*	This study
FA03	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*fadD*, P_{119}-*fadL*	This study
FA07	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*fadD*, P_{119}-*fadL*,Δ*sthA*	This study
FA09	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*fadD*, P_{119}-*fadL*,Δ*sthA*, P_{CPA1}-*pntAB*	This study
FA11	*E. coli* BW25113/F′, Δ*fadR*::P_{119}-*bt-ca*, P_{CPA1}-*fadD*, P_{119}-*fadL*, Δ*sthA*, P_{CPA1}-*pntAB*	This study
FA04	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*fadD*, P_{CPA1}-*sthA*	This study
FA06	*E. coli* BW25113/F′, Δ*fadR*, P_{CPA1}-*fadD*, Δ*pntA*	This study
HP01	FA01 carrying pXB1k-MCRNC	This study
HP02	FA03 carrying pXB1k-MCRNC	This study
HP03	FA03 carrying pXB1k-MCRCN	This study
HP04	FA03 carrying pSB1s-MCRCN	This study
HP05	FA07 carrying pSB1s-MCRCN	This study
HP06	FA09 carrying pSB1s-MCRCN	This study
HP07	FA09 carrying pSB1s-MCRCN, pLB1k-ACC	This study
HP08	FA11 carrying pSB1s-MCRCN, pLB1k-ACC	This study
HP21	FA04 carrying pSB1s-MCRCN	This study
HP22	FA06 carrying pSB1s-MCRCN	This study
Plasmid	**Description**	**Source**
pXB1k	araBAD promoter, p15A ori, Kan^r	Our laboratory
pSB1s	araBAD promoter, pSC101 ori, Str^r	Our laboratory
pLB1k	araBAD promoter, r6k ori, Kan^r	This study
pS95s	119 promoter, pSC101 ori, Str^r	Our laboratory
pS195s	119 promoter, pSC101 ori with temperature sensitive mutant, Str^r	Our laboratory
pS95s-GFP	pS95s containing EGFP gene	Our laboratory
pXB1k-MCRNC	pXB1k containing $mcr_{1–549}$ -rbs- $mcr_{550–1219}$ N940V K1106W S1114R	This study
pXB1k-MCRCN	pXB1k containing $mcr_{550–1219}$ N940V K1106W S1114R-rbs-$mcr_{1–549}$	This study
pSB1s-MCRCN	pSB1s containing $mcr_{550–1219}$ N940V K1106W S1114R-rbs-$mcr_{1–549}$	This study
pLB1k-ACC	pLB1k containing *accB*-rbs-*accC*-rbs-*accD*-rbs-*accA*	This study
pKD46	Temperature sensitive vector carrying Red recombinase, Amp^r	(Datsenko and Wanner, 2000)
pSL1k	pSC101 ori, Kan^r selective marker flanked by lox71 and lox66	Our laboratory
pSLC1k	CPA1 promoter, pSC101 ori, Kan^r selective marker flanked by lox71 and lox66	This study
pSL91k	119 promoter, pSC101 ori, Kan^r selective marker flanked by lox71 and lox66	This study
pSLT1k	tac promoter, pSC101 ori, Kan^r selective marker flanked by lox71 and lox66	This study
pSL911k-BC	P_{119}-*bt-ca*, pSC101 ori, Kan^r selective marker flanked by lox71 and lox66	This study
pS195s-Cre	pS195s containing Cre recombinase gene, Str^r	Our laboratory
pCP20	Temperature sensitive vector carrying FLP recombinase, Amp^r	(Datsenko and Wanner, 2000)

[a] CGSC: Coli Genetic Stock Center.

expression of *acc* and *mcr*. The carbon source was switched from glucose to FAs when the OD_{600} reached 50. For batch fermentation, cultures were harvested after induction and washed twice, and then transferred into 2 L M9 FAs medium supplemented with 0.3 g/L yeast exact. For fed-batch fermentation, cultures were directly fed with FAs medium supplemented with 0.1 g/L yeast extract when DO concentration reached 50%. The pH was controlled at 7.0 via automated addition of 1 M $NaHCO_3$, and an extra 20 mM $NaHCO_3$ was added every 12 h. The air flow and stirring rate were set to 0.2 L/min and 600 rpm, respectively. The DO content was controlled in the range of 5–10%.

2.7. Analytical methods

The concentrations of extracellular metabolites were analyzed by high performance liquid chromatography (HPLC, LC 20A LabSolutions, Shimadzu Corp., Kyoto, Japan). Briefly, a 1 mL sample was freeze-thawed twice, centrifuged at 13,000 ×*g* for 10 min, filtered through a 0.22 μM PES membrane (Jinteng, Tianjin, China) and eluted through a 300 mm × 7.8 mm Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 55 °C using 5 mM H_2SO_4 (flow rate, 0.6 mL/min) in 30 min. We detected 3HP and other organic acids using a refractive index (RI) detector.

The concentrations of intracellular metabolites were determined by LC-MS/MS (liquid chromatography–mass spectrometry). Cells were collected by centrifugation at 13,000 ×*g* for 10 min, washed twice with ice-cold 0.85% NaCl solution, suspended in 400 μL (OD_{600} = 250) of 80% (vol/vol) methanol (prechilled to −80 °C), and sonicated as described previously (Cui et al., 2017). The LC-MS/MS system consisted of a Dionex Ultimate 3000 UPLC (Dionex, Sunnyvale, CA, USA) coupled to a TSQ Quantiva Ultra triple-quadrupole mass spectrometer (Thermo Fisher, Waltham, MA, USA) equipped with a heated electrospray ionization (HESI) probe. Extracts were separated on a Luna C5 column (2.0 × 100 mm, 5 μm; Phenomenex, Torrance, CA, USA). The binary solvent system consisted of mobile phase A (0.2% (v/v) HFIP and 0.04% (v/v) TEA in water) and mobile phase B (isopropanol). A 15-min gradient with a flow rate of 250 μL/min was applied as follows: 0–1.2 min at 2% B, 1.2–9 min at 2–98% B, 9–11.9 min at 98% B, 11.9–12 min at 98–2% B, 12–15 min at 2% B. The column chamber and sample tray were held at 35 °C and 10 °C, respectively. Data were acquired by selected reaction monitoring (SRM) in the negative ion mode. Both the precursor and fragment ions were collected with a resolution of 0.7 FWHM. Data analysis and quantification were performed using Xcalibur 3.0.63 software (Thermo Fisher).

Free FAs were quantified using a gas chromatograph (GC; 7890A GC system, Agilent Technologies, Palo Alto, USA) equipped with a flame ionization detector. Each 200-μL FAs sample was converted to FA methyl esters (FAMEs) with 800 μL 1.8 M H_2SO_4 in 90% (V/V) methanol at 70 ± 1 °C for 20 min. Then, 500 μL hexane was added to the cooled reaction mixture, followed by vigorous vortexing to extract FAMEs. The mixture was centrifuged at maximum speed to separate aqueous and organic layers, and then 200 μL of the hexane layer containing the products was transferred to GC vials for quantification. The GC analysis was carried out with an HP-88 column (Agilent Technologies) with 0.2-μm film thickness, 0.25 mm diameter, and 60 m length. The GC program was as follows: initial oven temperature of 150 °C for 5 min, followed by ramping up to 170 °C at a rate of 3 °C/min, and held at that

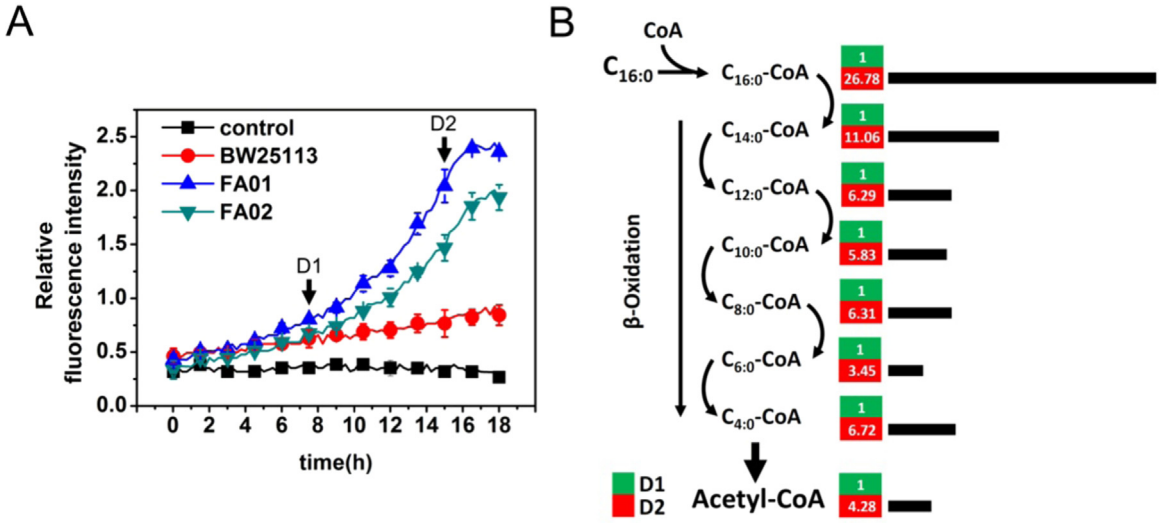


Fig. 2. Analysis of fatty acids (FAs) utilization in *Escherichia coli*. A. Growth curves in FAs-containing medium as measured by GFP fluorescence. B. Intracellular acyl-CoAs in strain FA01. Quantities in green boxes indicate relative amounts of acyl-CoAs at D1 stage in Fig. 2A; quantities in red boxes indicate relative amounts of acyl-CoAs at D2 stage in Fig. 2A; black bars on right indicate ratio of acyl-CoA amounts between D2 and D1 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

temperature for 5 min. The temperature was then increased up to 210 °C at a rate of 3 °C/min, and held at that temperature for 5 min. All data were obtained from three replicates.

3. Results and discussion

3.1. Construction of primary strains for FA utilization

To develop an ideal strain that could efficiently use FAs, we first modified FA-utilization targets. Derepression of *fadR* and constitutive expression of *atoSC* have been reported to facilitate FAs utilization in *E. coli* (Dellomonaco et al., 2010; Rhie and Dennis, 1995). Accordingly, strain FA01 was obtained by knocking out *fadR*, which encodes a major regulator that represses the transcription of FA transport and β-oxidation enzymes. The short chain FA utilization-related *atoSC* operon of FA01 was further modified by replacing its native promoter with the strong CPA1 promoter, yielding strain FA02.

The strains were inoculated into M9 FAs medium, and the growth curves were recorded by monitoring GFP intensity over 48 h. As shown in Fig. 2A, in the first 24 h of culture, the GFP intensity of wild-type *E. coli* only slightly increased, indicating that wild-type *E. coli* could not efficiently use palmitic acid. In contrast, both FA01 and FA02 were able to utilize palmitic acid efficiently, as shown by the significant increases in GFP intensity. The growth curve of FA02 was similar to that of FA01, suggesting that the deletion of *fadR* may have been more important for FA utilization than the increase in *atoSC* expression. We also modified the expression of *atoSC* by introducing constitutive promoters, but this did not improve FAs utilization (data not shown). Therefore, only strain FA01 was used for further engineering.

A lag phase of about 12 h was observed for both FA01 and FA02. We compared the levels of acyl-CoA intermediates between the lag phase (D1) and the mid-exponential growth phase (D2). The level of long-chain fatty acyl-CoA (C_{16}-CoA), which is the immediate intermediate after FA uptake and activation, was significantly higher in D2 than in D1 (Fig. 2B). This suggested that a cellular response involving FA utilization was highly induced after the lag phase. These results spurred us to analyze the exact genetic response and elucidate other key engineering targets.

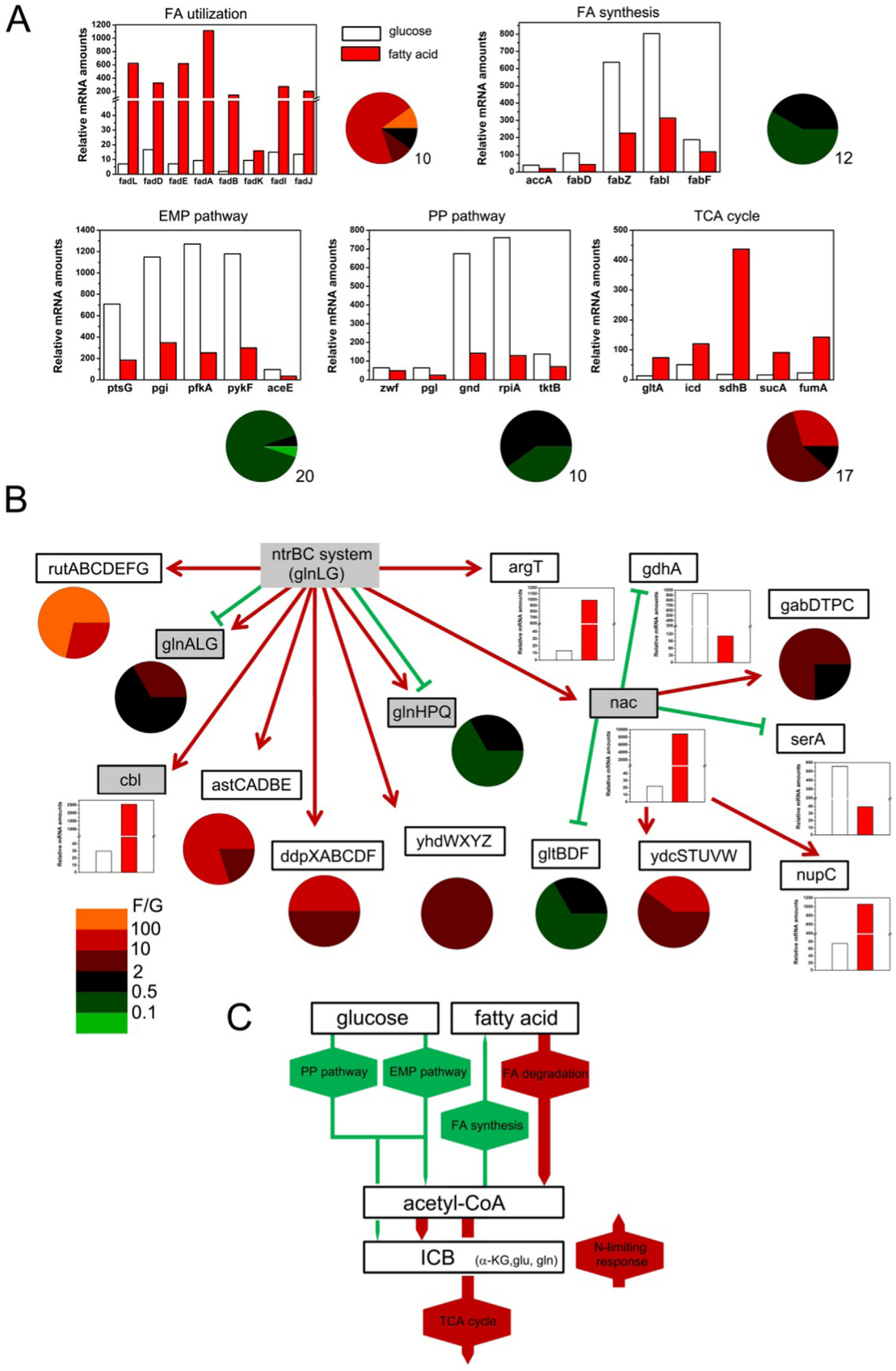
3.2. Transcriptomic analysis of strains grown with FAs

To systematically analyze the global gene expression response during adaptation to growth with FAs as the substrate, we compared the transcriptomes of FA01 between FA-substrate and glucose-substrate conditions at the mid-exponential growth phase. In total, 643 genes were differentially expressed by at least 2-fold ($p < 0.05$) between the two conditions. Among them, 354 genes showed at least 2-fold increases in expression in cells grown with FA as compared with those grown with glucose. 54 showed more than 10-fold higher expression and 12 showed more than 100-fold higher expression with FA substrate than with glucose substrate. The other 244 genes showed at least 2-fold lower expression levels with FA as the substrate than with glucose as the substrate, and 34 of them showed more than 10-fold decreases in their levels.

The differentially expressed genes were involved in a wide range of cellular metabolic processes. As expected, even when the repressor *fadR* gene had been deleted from the FA01 strain, most of the genes related to FA uptake and β-oxidation were expressed at significantly higher levels (15- to 120-fold) with FAs as the substrate than with glucose as the substrate (Fig. 3A). This result indicated that there might be additional global regulators that regulate FA utilization in FA-substrate conditions. Most of the genes related to FA biosynthesis were expressed at lower levels with FAs as the substrate than with glucose as the substrate.

The differentially expressed genes involved in central carbon metabolism are shown in Fig. 3B. When FAs were supplied as the substrate, most genes related to glycolysis and the pentose phosphate pathway were down-regulated, while most genes related to the TCA cycle were up-regulated. These results were consistent with the metabolic profile of FAs utilization. That is, FAs are utilized via the formation of the intermediate acetyl-CoA, thus bypassing the Embden Meyerhof Parnas pathway and the pentose phosphate pathway.

Notably, a series of genes under the control of the nitrogen regulator ntrBC system were highly up/down-regulated when cells were grown with FAs as the substrate (Fig. 3B). Nac, the second nitrogen regulator activated by ntrBC, and Cbl, a sulfate regulator downstream of nac, were up-regulated by more than 100-fold with FA as the substrate, compared with glucose as the substrate. Most genes in NtrBC-activated operons, including the *rutABCDEFG* operon (encoding pyrimidine degradation enzymes), the *astCADBE* operon (encoding arginine catabolic



(caption on next page)

Fig. 3. Comparative transcriptomic analysis between strain FA01 grown with glucose and FA01 grown with fatty acids (FAs) as substrate. Column charts indicate difference of single gene or selected gene, pie charts show difference of total number of genes (total number of genes is shown in lower right corner of pie charts; colors indicate the relative -fold difference in mRNA levels between FA substrate and glucose substrate (F/G); red and green lines indicate that the mRNA level is up-regulated and down-regulated, respectively, in FA conditions. A. Differentially expressed genes related to FA metabolism and central carbon metabolism; B. Differentially expressed genes related to N-limitation response; names of gene/gene clusters are shown in boxes (regulators of N-limitation response are shown in gray boxes); C. Summary of transcriptional differences between glucose conditions and FA conditions, ICB, intermediates for cellular biosynthesis (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

pathway enzymes), the *ddpXABCDF* operon (encoding enzymes that convert murein to D-ala-D-ala), and the *argT* and *yhdXWYZ* operons (encoding ABC transporter components) were expressed at 2-fold to more than 100-fold higher levels with FAs as the substrate than with glucose as the substrate. Nac-responsive genes, such as *gdhA*, *gltBDF*, *serA*, *nupC*, and *ydcSTUVW*, showed similar patterns of up-regulation.

NtrBC system is the main regulator involved in the response to changing nitrogen conditions. Activation of NtrBC-regulated genes is usually associated with the starvation of ammonia, the preferred nitrogen source (Gyaneshwar et al., 2005; Reitzer, 2003; Zimmer et al., 2000). The above results suggest that ammonia, the inorganic type nitrogen source, could not be efficiently assimilated when FAs were supplied as the substrate. We speculated that this might be due to the inefficient supply of ammonia assimilation-related intermediates (e.g., α-ketoglutarate) because of the insufficient anaplerotic reaction towards the TCA cycle from glycolysis when FAs were used as the substrate. Our results were also consistent with the reports that FAs metabolism is closely related to nitrogen regulation and condition (Gerhardt et al., 2015; Liu et al., 2018b; Xu et al., 2016). Thus, when FAs are supplied as the substrate, alternative nitrogen sources will be essential to maintain the normal physiological function of cells.

3.3. Efficient utilization of FAs by E. coli

According to the results of the transcriptomic analysis, we enhanced the expression of several FA uptake and β-oxidation pathway genes by replacing the native promoters with strong promoters. The native promoters of *fadE, fadD, fadBA*, and *fadL* were replaced with constitutive promoters (Table S3) of three different strengths, namely P_{CPA1} (strong promoter), P_{119} (medium-strong promoter), and P_{tac} (medium promoter). The results indicated that FAs utilization was improved by replacing the native promoter of *fadD* with the P_{CPA1} promoter (Fig. 4). Replacing the *FadL* promoter also improved FA-utilization, but only when the medium-strong promoter was used. This result was consistent with the results of the metabolite analysis (Fig. 2B), which suggested that FA-uptake might be the limiting step in FAs utilization.

To further evaluate the FA utilization of the resulting strains, the whole-cell bioconversion of FA substrate in M9 salt FAs medium was monitored for 2–5 h. In addition, according to the results of the transcriptomic analysis, we compared the FA utilization efficiency of the FA01 and FA03 strains in media containing different organic nitrogen sources. The results showed that the addition of organic nitrogen sources during the transformation phase significantly increased FAs utilization, and the addition of 0.3 g/L yeast extract produced the best results (further increasing concentration of yeast extract could not increase FAs utilization, data not shown). Enhancement of *fadD* and *fadL* also improved the FAs utilization ability of the strains. Strain FA03, in which both *fadD* and *fadL* were enhanced, efficiently used FAs at a rate of 1.6 $g\,L^{-1}\,h^{-1}$20 OD^{-1}.

3.4. Optimized expression of enzymes involved in 3HP production module

The 3HP production module was first constructed by introducing *mcrC* and *mcrN* (modified genes from *Chloroflexus aurantiacus*, (Liu et al., 2016)) into a p15A-derived plasmid, resulting in pXB1k-MCRNC. Strain HP01, obtained by introducing pXB1k-MCRNC into FA01, produced 3HP to a concentration of 0.34 ± 0.05 g/L after bioconversion for 20 h. Strain HP02, which was obtained by replacing FA01 with FA03, produced 3HP to a concentration of about 0.61 ± 0.12 g/L.

Since the expression level of MCRN and MCRC has been shown to be crucial to 3HP biosynthesis pathway (Liu et al., 2016), we optimized the expression of *mcrC* and *mcrN* by changing the plasmid copy number and gene order. Placement of *mcrC* into the first site in the operon (strain HP03) increased 3HP production to 1.57 ± 0.12 g/L. The result of SDS-PAGE confirmed this modification could decrease the amounts of MCRN (Fig. 5. C).A further replacement of the p15A-derived plasmid with the low copy number plasmid pSC101 (strain HP04) resulted in the production of 2.12 ± 0.49 g/L of 3HP. However, chromosomal expression of multi-copy of both *mcrN* and *mcrC* was not adequate for higher 3HP production (data not shown). Taken together, lower expression levels of pathway genes and use of low-copy-number plasmid might be more favorable for 3HP production.

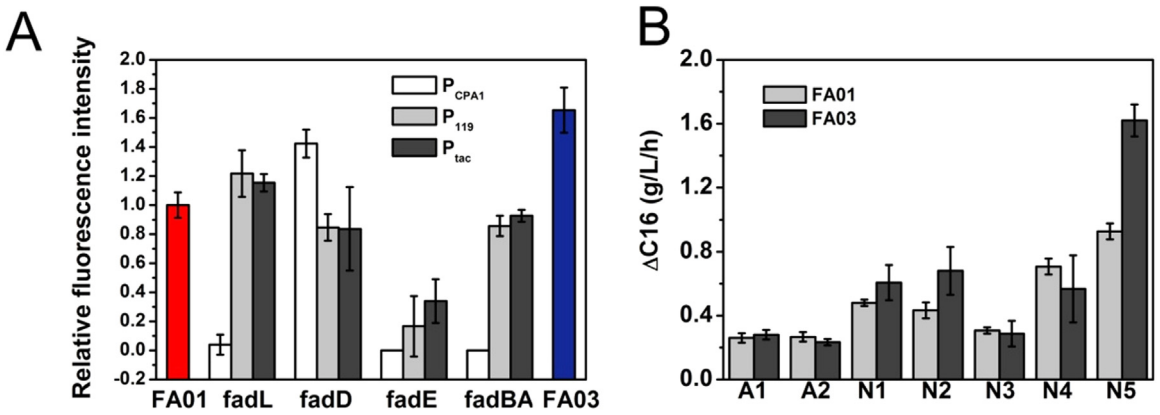


Fig. 4. Metabolic engineering and optimization of fermentation conditions for efficient fatty acids (FAs) utilization. A. Effects of replacing promoters of genes related to FA utilization on cell growth in FA conditions (measured by GFP fluorescence after culture for 15 h). B. Effects of different N conditions on FA utilization. A1, 2 mM ammonium salt; A2, 10 mM ammonium salt; N1, 2 mM glutamate; N2, 2 mM α-ketoglutarate; N3, 2 mM glutamine; N4, 2 mM glutamate/α-ketoglutarate/glutamine; N5, 0.3 g/L yeast extract.

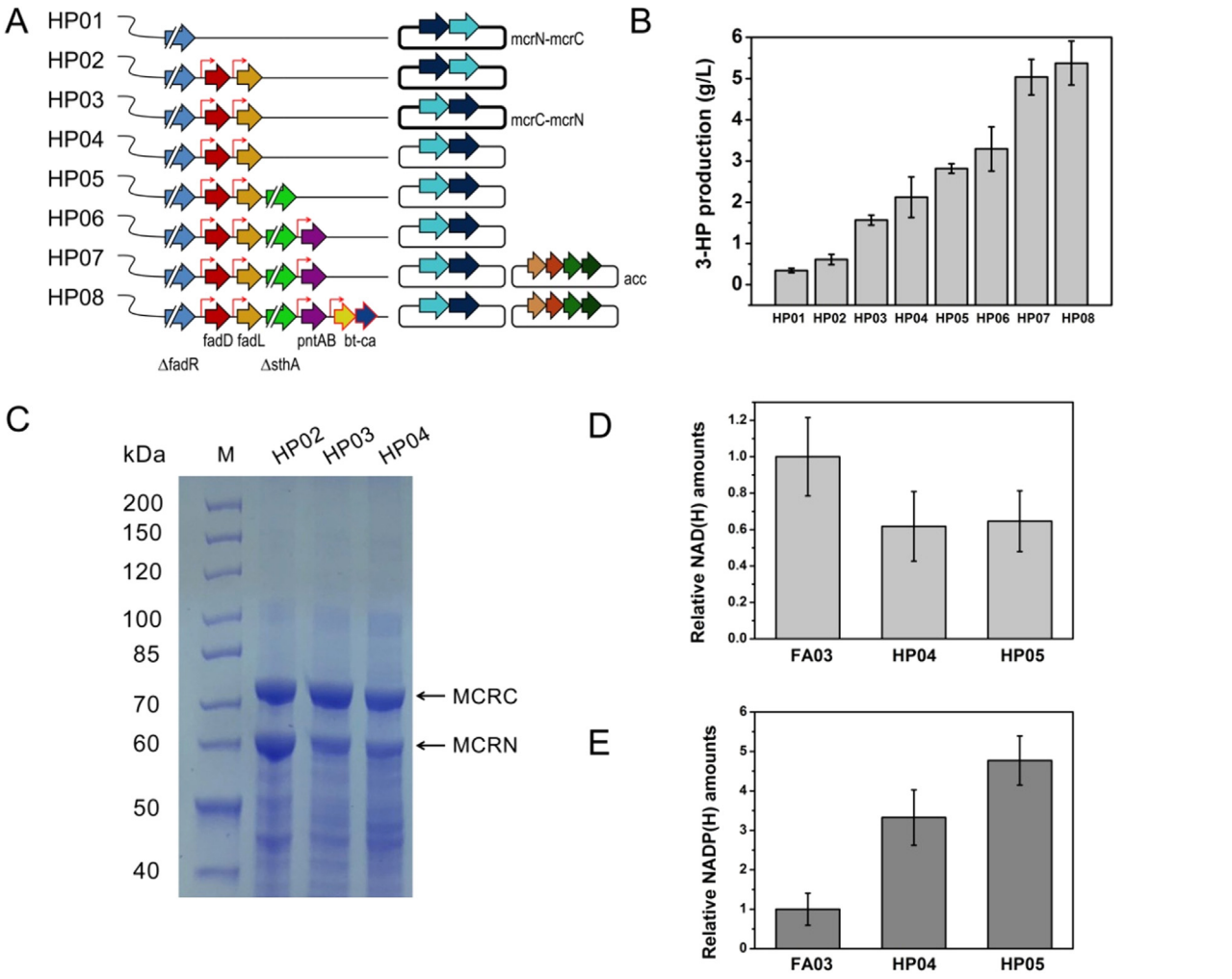


Fig. 5. Metabolic engineering for 3HP production from FAs. A. Construction of different strains. Lines on left indicate genomic modification targets; gene icons with slashes indicate deleted genes; gene icons with red arrows indicate native promoters of genes replaced by strong promoters; gene icons with red box and arrow indicate heterologous genes; cycles on the right indicate plasmid expression targets; thick circles indicate medium copy-number plasmids; thin circles indicate low copy-number plasmids; B. 3HP production by eight different strains; C. SDS-PAGE analysis of different strains; D. relative intracellular NAD(H) levels of different strains; E. relative intracellular NADP(H) levels of different strains (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.5. Balance of reducing power by engineering SthA and PntAB

As the production of 3HP was still relatively low, we then investigated the limit factors by the evaluation of relative levels of metabolic intermediates and cofactors that related to the 3HP biosynthesis pathway. The levels of intracellular intermediates of β-oxidation and TCA cycle, 3HP biosynthesis precursor malonyl-CoA, and cofactors including ATP, FAD(H_2), NAD(H), and NADP(H), were compared between strain FA03 and HP04. No obvious deference of intermediates levels was found (data not shown), except for that of NAD(H) and NADP (H) level. It was found when 3HP production module was introduced into FA03 (resulting in HP04), the NADP(H) level was increased by about 3.3 folds. Meanwhile, the NAD(H) level was decreased by about 40% (Fig. 5. D-E). Considering the fact that β-oxidation of FAs generates NADH, and the 3HP synthesis module requires NAPDH, it is most likely that the strain undergoes an adaptive regulation of NAD(H)-NADP(H) conversion to sustain 3HP biosynthesis. Thus we speculated that a large amount of NADP(H) was necessary for higher 3HP production.

Two enzymes were involved in the conversion between NADH and NADPH: SthA (soluble pyridine nucleotide transhydrogenase) possess the activity to catalyze the formation of NADH from NADPH, while the function of PntAB (membrane-bound proton-translocating transhydrogenase) is generating NADPH from NADH (Sauer et al., 2004). The *sthA* gene was first knocked out in strain HA04. As expect, the deletion of *sthA* (strain HP05) increased 3HP production to 2.82 ± 0.11 g/L. NADP(H) level of HP05 was also increased by 44% compared with that of HP04. A serious of modification of *sthA* and *pntAB*, were also carried out by either knocked out or enhanced using different strategies (some of them summarized in Table S1). The results indicated further chromosomal enhancement of *pntAB* (strain HP06) expression increased 3HP production to 3.29 ± 0.53 g/L. These results confirmed the importance of the reducing power supply for 3HP production (Fig. 5).

3.6. Improvement of malonyl-CoA supply by engineering ACC and CCM

After bioconversion of HP06 strain for 20 h, the FA-source has been exhausted, which is corresponding to relative low yield of 3HP (0.32 g/g FAs). This implied further effort should be made to direct more metabolic flux into 3HP biosynthesis pathway. The synthesis of 3HP requires the assimilation of bicarbonate with acetyl-CoA by the ACC carboxyltransferase complex to generate the precursor malonyl-CoA. To improve the efficiency of this step, we further overexpressed genes encoding the ACC complex using a second low-R6k-derived plasmid

containing the araBAD promoter, yielding strain HP07. The 3HP production of HP07 was improved to 5.04 ± 0.43 g/L. Meanwhile, it was reported that *E. coli* assimilated CO_2/bicarbonate at low efficiency (Xiao et al., 2017), which could further limit the supply of malonyl-CoA. To address this issue, carbon-concentrating mechanism (CCM), including the bicarbonate transporter (*BT*) gene from *Synechococcus* sp. and carbonic anhydrase (*CA*) gene from *Anabaena* sp. PCC 7120 were introduced and chromosomally overexpressed. The resulting strain HP08 produced 3HP to a final concentration of 5.38 ± 0.53 g/L (with a yield of 0.54 g/g FAs in flask). This is the optimal strain for 3HP production from FAs feedstock so far.

3.7. Fermentation production of 3HP

Next, we intended to improve the yield by bioconversion process optimization. Both batch fermentation and fed-batch fermentation to produce 3HP were carried out at the 5-L fermenter level. The fermentation conditions were determined based on two considerations: first, the TCA cycle should be repressed to increase acetyl-CoA supply for 3HP biosynthesis; and second, the respiration level should be appropriate to consume excess reducing power and generate ATP for CO_2 fixation. Through successive optimizations, microaerobic fermentation conditions were established. In the batch fermentation, strain FA08 was first cultured in LB medium to induce enzymes, and then the medium was replaced with medium containing 10 g/L palmitic acid, and fermentation proceeded for 3 h. This bioconversion produced 3HP with a titer of 9.35 g/L and a yield of 1.56 g/g FAs, about 62% of its theoretical yield. In the fed-batch fermentation, palmitic acid was supplied with 200 g/L palmitic acid (emulsified with 40 g/L Brij 58) at a final concentration of about 2 g/L when the DO concentration reached 50%. After fed-batch fermentation for 46 h, totally 26.95 g/L palmitic acid was added, the final volume of the culture reached 2.58 L and the final 3HP concentration reached about 52 g/L (Fig. 6. A).

3.8. production of 3HP from gutter oil feedstock

To evaluate the possibility of using cheap FA feedstock to produce 3HP, gutter oil and soybean oil were used as raw material for the production of 3HP at flask level. The major gutter oil composition is FAs with different chain length and saturation in triglyceride form, which is similar to soybean oil. Both gutter oil and soybean oil cannot be used initially by HP08. Thus, during bioconversion process, 15 mg/L lipase was added at 0, 7 and 21 h. The results indicated 3.4 ± 0.22 g/L and 4.4 ± 0.28 g/L 3HP production were accumulated, respectively, from gutter oil and soybean oil after bioconversion for 45 hrs (Fig. 6. B).

It seems gutter oil can be utilized efficiently in the initial stage of bioconversion, as 2.7 g/L (about 80% of final production) and 3.2 g/L (about 95% of final production) of 3HP had been produced after bioconversion for 7 and 21 hrs, respectively. The 3HP productivity (7 h) of gutter oil was 0.38 g/L/h, which is higher than that of palmitic acid (0.29 g/L/h for 7 h). However, no obviously higher 3HP production was observed after 21 hrs. This is most likely due to the various FAs composition of gutter oil, and the ability to use different FAs should be further improved.

3.9. Discussion

In this study, we successfully develop a novel technique route for 3HP production using FAs as feedstock. This route possess advantages in several aspects: (1) avoids loss of carbon during 3HP biosynthesis; (2) efficiently supplies ATP for CO_2 fixation; (3) efficiently supplies reducing power for 3HP biosynthesis; (4) provides solution for the utilization of waste feedstock.

E. coli does not naturally utilize FAs as feedstock. A previously reported *E. coli* variant (Dellomonaco et al., 2010) also could not efficiently utilize FAs in our study (Fig. 2A, corresponding to FA02). Therefore, the development of an efficient *E. coli* strain utilizing FAs is not only the key step for this work, but is also important for the construction of a competitive and novel biomass resource route for the production of valuable chemicals. By systematic metabolic optimization of FA-utilization pathways, mainly FA uptake and global regulation, we have developed an optimized *E. coli* strain that can efficiently use FAs as feedstock. The efficiency of the bioconversion of FA raw materials was also significantly improved by optimizing the fermentation conditions. These developments have greatly shortened the fermentation period compared with that reported previously (Dellomonaco et al., 2010), making it possible to use FAs as raw materials at the industrial level.

Our study has elucidated the potential of this strain to efficiently produce 3HP, an important platform chemical, from FAs feedstock. In the recent decade, 3HP was reported to be biosynthesized mainly using glucose or glycerol as feedstocks (de Fouchecour et al., 2018). The theoretical yields of all these reported routes are around 1 g/g glucose/glycerol (de Fouchecour et al., 2018). In this study, 3HP can be produced from FAs feedstock with a yield of 1.56 g/g FAs at 5 L bioreactor level. This yield was much higher than those from other feedstocks reported previously in the literature, in which the yield of 3HP around 0.2–0.6 g/g (de Fouchecour et al., 2018). Our study also provides the possibility to produce 3HP from waste oil feedstock. The high product yield and low cost of feedstock (waste oil) make FAs route become the most competitive route for 3HP production.

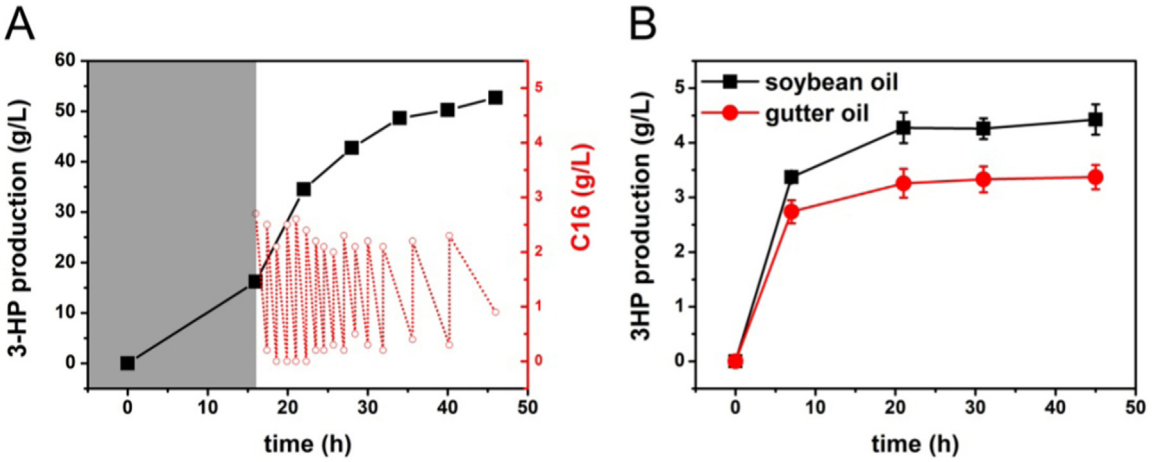


Fig. 6. A. Fed-batch fermentation in a 5-L system for 3HP production from FAs medium. Gray area indicates protein induction stage; black line indicates 3HP production; red dot line indicated FAs fed concentration; B. 3HP production from gutter oil and soybean oil by strain HP08 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

In this study, all key limiting factors for 3HP production, including the supply of precursors and NADPH, have been addressed. Meanwhile, our strains also have some adaptive advantages. For example, the expression levels of FA biosynthesis-related genes were intrinsically low in our strains (Fig. 2B), which might result in the high malonyl-CoA level for 3HP production. As the next step, we intend to further optimize the flux of the biosynthesis pathway, regenerating $FADH_2$ to increase the reducing power supply and further improve the yield of 3HP. Together, the results of the present study demonstrate an effective technological route to use FAs as raw materials to produce not only 3HP, but also other important chemicals.

Acknowledgments

This work was supported by the Key Research Program of the Chinese Academy of Sciences (No. ZDRW-ZS-2016-3-1) and National Natural Science Foundation of China (No. 31670051). We are grateful for the support of the Metabolomics Facility at the Technology Center for Protein Sciences of Tsinghua University. We thank Jennifer Smith, PhD, from the Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2018.10.003.

References

Antonovsky, N., Gleizer, S., Noor, E., Zohar, Y., Herz, E., Barenholz, U., Zelcbuch, L., Amram, S., Wides, A., Tepper, N., Davidi, D., Bar-On, Y., Bareia, T., Wernick, D.G., Shani, I., Malitsky, S., Jona, G., Bar-Even, A., Milo, R., 2016. Sugar synthesis from CO_2 in *Escherichia coli*. Cell 166, 115–125.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H., 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2 (2006 0008).

Borodina, I., Kildegaard, K.R., Jensen, N.B., Blicher, T.H., Maury, J., Sherstyk, S., Schneider, K., Lamosa, P., Herrgard, M.J., Rosenstand, I., Oberg, F., Forster, J., Nielsen, J., 2015. Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in Saccharomyces cerevisiae via beta-alanine. Metab. Eng. 27, 57–64.

Chae, T.U., Choi, S.Y., Kim, J.W., Ko, Y.S., Lee, S.Y., 2017. Recent advances in systems metabolic engineering tools and strategies. Curr. Opin. Biotechnol. 47, 67–82.

Chang, A.S., Sherazi, S.T., Kandhro, A.A., Mahesar, S.A., Chang, F., Shah, S.N., Laghari, Z.H., Panhwar, T., 2016. Characterization of palm fatty acid distillate of different oil processing industries of Pakistan. J. Oleo Sci. 65, 897–901.

Choi, K.Y., Wernick, D.G., Tat, C.A., Liao, J.C., 2014. Consolidated conversion of protein waste into biofuels and ammonia using Bacillus subtilis. Metab. Eng. 23, 53–61.

Chubukov, V., Mukhopadhyay, A., Petzold, C.J., Keasling, J.D., Martin, H.G., 2016. Synthetic and systems biology for microbial production of commodity chemicals. NPJ Syst. Biol. Appl. 2, 16009.

Cui, Q., Zhou, F., Liu, W., Tao, Y., 2017. Avermectin biosynthesis: stable functional expression of branched chain alpha-keto acid dehydrogenase complex from Streptomyces avermitilis in Escherichia coli by selectively regulating individual subunit gene expression. Biotechnol. Lett. 39, 1567–1574.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97, 6640–6645.

Dellomonaco, C., Rivera, C., Campbell, P., Gonzalez, R., 2010. Engineered respiro-fermentative metabolism for the production of biofuels and biochemicals from fatty acid-rich feedstocks. Appl. Environ. Microbiol. 76, 5067–5078.

Erb, T.J., 2011. Carboxylases in natural and synthetic microbial pathways. Appl. Environ. Microbiol. 77, 8466–8477.

Feng, Y., Cronan, J.E., 2012. Crosstalk of Escherichia coli FadR with global regulators in expression of fatty acid transport genes. PloS One 7, e46275.

de Fouchecour, F., Sanchez-Castaneda, A.K., Saulou-Berion, C., Spinnler, H.E., 2018. Process engineering for microbial production of 3-hydroxypropionic acid. Biotechnol. Adv.

Gerhardt, E.C., Rodrigues, T.E., Muller-Santos, M., Pedrosa, F.O., Souza, E.M., Forchhammer, K., Huergo, L.F., 2015. The bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase. Mol. Microbiol. 95, 1025–1035.

Green, M.R., Sambrook, J., 2012. Molecular Cloning: A Laboratory Manual, 4th edn. Cold Spring Harbor Laboratory Press, New York.

Gustavsson, M., Lee, S.Y., 2016. Prospects of microbial cell factories developed through systems metabolic engineering. Microb. Biotechnol. 9, 610–617.

Gyaneshwar, P., Paliy, O., McAuliffe, J., Popham, D.L., Jordan, M.I., Kustu, S., 2005. Sulfur and nitrogen limitation in Escherichia coli K-12: specific homeostatic responses. J. Bacteriol. 187, 1074–1090.

Hernandez, N., Williams, R.C., Cochran, E.W., 2014. The battle for the "green" polymer. Different approaches for biopolymer synthesis: bioadvantaged vs bioreplacement. Org. Biomol. Chem. 12, 2834–2849.

Hu, G., Zhou, J., Chen, X., Qian, Y., Gao, C., Guo, L., Xu, P., Chen, W., Chen, J., Li, Y., Liu, L., 2018. Engineering synergetic CO_2-fixing pathways for malate production. Metab. Eng. 47, 496–504.

Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Curr. Opin. Biotechnol. 42, 30–39.

Kildegaard, K.R., Jensen, N.B., Schneider, K., Czarnotta, E., Ozdemir, E., Klein, T., Maury, J., Ebert, B.E., Christensen, H.B., Chen, Y., Kim, I.K., Herrgard, M.J., Blank, L.M., Forster, J., Nielsen, J., Borodina, I., 2016. Engineering and systems-level analysis of Saccharomyces cerevisiae for production of 3-hydroxypropionic acid via malonyl-CoA reductase-dependent pathway. Microb. Cell Factor. 15, 53.

Kim, J.J., Battaile, K.P., 2002. Burning fat: the structural basis of fatty acid beta-oxidation. Curr. Opin. Struct. Biol. 12, 721–728.

Kim, K., Kim, S.K., Park, Y.C., Seo, J.H., 2014. Enhanced production of 3-hydroxypropionic acid from glycerol by modulation of glycerol metabolism in recombinant Escherichia coli. Bioresour. Technol. 156, 170–175.

Kircher, M., 2015. Sustainability of biofuels and renewable chemicals production from biomass. Curr. Opin. Chem. Biol. 29, 26–31.

Kluts, I.N., Brinkman, M.L.J., de Jong, S.A., Junginger, H.M., 2017. Biomass resources: agriculture. Adv. Biochem. Eng./Biotechnol.

Liang, S., Liu, Z., Xu, M., Zhang, T., 2013. Waste oil derived biofuels in China bring brightness for global GHG mitigation. Bioresour. Technol. 131, 139–145.

Liu, C., Ding, Y., Zhang, R., Liu, H., Xian, M., Zhao, G., 2016. Functional balance between enzymes in malonyl-CoA pathway for 3-hydroxypropionate biosynthesis. Metab. Eng. 34, 104–111.

Liu, C., Ding, Y., Xian, M., Liu, M., Liu, H., Ma, Q., Zhao, G., 2017. Malonyl-CoA pathway: a promising route for 3-hydroxypropionate biosynthesis. Crit. Rev. Biotechnol. 37, 933–941.

Liu, R., Liang, L., Choudhury, A., Bassalo, M.C., Garst, A.D., Tarasava, K., Gill, R.T., 2018a. Iterative genome editing of Escherichia coli for 3-hydroxypropionic acid production. Metab. Eng. 47, 303–313.

Liu, X.X., Shen, M.J., Liu, W.B., Ye, B.C., 2018b. GlnR-mediated regulation of short-chain fatty acid assimilation in mycobacterium smegmatis. Front. Microbiol. 9, 1311.

Luo, H., Zhou, D., Liu, X., Nie, Z., Quiroga-Sanchez, D.L., Chang, Y., 2016. Production of 3-hydroxypropionic acid via the propionyl-CoA pathway using recombinant Escherichia coli strains. PloS One 11, e0156286.

Reitzer, L., 2003. Nitrogen assimilation and global regulation in Escherichia coli. Annu. Rev. Microbiol. 57, 155–176.

Rhie, H.G., Dennis, D., 1995. Role of fadR and atoC(Con) mutations in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant pha+ Escherichia coli. Appl. Environ. Microbiol. 61, 2487–2492.

Sanford, K., Chotani, G., Danielson, N., Zahn, J.A., 2016. Scaling up of renewable chemicals. Curr. Opin. Biotechnol. 38, 112–122.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., Fischer, E., 2004. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli. J. Biol. Chem. 279, 6613–6619.

Seok, J.Y., Yang, J., Choi, S.J., Lim, H.G., Choi, U.J., Kim, K.J., Park, S., Yoo, T.H., Jung, G.Y., 2018. Directed evolution of the 3-hydroxypropionic acid production pathway by engineering aldehyde dehydrogenase using a synthetic selection device. Metab. Eng. 47, 113–120.

Song, C.W., Kim, J.W., Cho, I.J., Lee, S.Y., 2016. Metabolic engineering of Escherichia coli for the production of 3-hydroxypropionic acid and malonic acid through beta-alanine route. ACS Synth. Biol. 5, 1256–1263.

Stephanopoulos, G., 2007. Challenges in engineering microbes for biofuels production. Science 315, 801–804.

Studier, F.W., 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. 41, 207–234.

Thomason, L.C., Costantino, N., Court, D.L., 2007. E. coli genome manipulation by P1 transduction. Curr. Protoc. Mol. Biol (Chapter 1, Unit 1 17).

Werpy, T., Peterson, G., 2004. Top Value Added Chemicals from Biomass. Office of Energy Efficiency and Renewable Energy. US Department of Energy, Washington D.C.

Wheeldon, I., Christopher, P., Blanch, H., 2017. Integration of heterogeneous and biochemical catalysis for production of fuels and chemicals from biomass. Curr. Opin. Biotechnol. 45, 127–135.

Xiao, M., Zhu, X., Bi, C., Ma, Y., Zhang, X., 2017. Improving succinate productivity by engineering a cyanobacterial CO_2 concentrating system (CCM) in Escherichia coli. Biotechnol. J. 12.

Xu, P., Qiao, K., Ahn, W.S., Stephanopoulos, G., 2016. Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. Proc. Natl. Acad. Sci. USA 113, 10848–10853.

ZERO, 2016. Palm Fatty Acid Distillate (PFAD) in biofuels. ZERO and Rainforest Foundation Norway. ⟨https://visionpdf.com/palm-fatty-acid-distillate-pfad-in-biofuels.html⟩, (Accessed 25 May 2018).

Zhao, L., Lin, J., Wang, H., Xie, J., Wei, D., 2015. Development of a two-step process for production of 3-hydroxypropionic acid from glycerol using Klebsiella pneumoniae and Gluconobacter oxydans. Bioprocess Biosyst. Eng. 38, 2487–2495.

Zimmer, D.P., Soupene, E., Lee, H.L., Wendisch, V.F., Khodursky, A.B., Peter, B.J., Bender, R.A., Kustu, S., 2000. Nitrogen regulatory protein C-controlled genes of Escherichia coli: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. USA 97, 14674–14679.

Zollinger, M., 2015. In China, the potential for harnessing the power of waste cooking oil. Yale Environment Review. ⟨https://environment-review.yale.edu/china-potential-harnessing-power-waste-cooking-oil-0⟩, (Accessed 25 May 2018).