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ORIGINAL ARTICLE

Butanol production from glycerol by recombinant *Escherichia coli*

Pengpeng Zhou · Yan Zhang · Pixiang Wang · Jingli Xie · Qin Ye

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Abstract *Escherichia coli* MG1655 (DE3) with the ability to synthesize butanol from glycerol was constructed by metabolic engineering. The genes *thil*, *adhe2*, *bcs* operon (crt, bcd, etfB, etfA, and hbd) were cloned into the plasmid vectors, pETDuet-1 and pACYCDuet-1, then the two resulting plasmids, pACYC-thl-bcs and pET-adhe2, were transferred to *E. coli*, and the recombinant strain was able to synthesize up to 18.5 mg/L butanol on a glycerol-containing medium. After the glycerol transport protein gene *GlpF* was expressed, the butanol production was improved to 22.7 mg/L. The competing pathway of byproducts, such as ethanol, succinate, and lactate, was subsequently deleted to improve the 1-butanol production to 97.9 mg/L. Moreover, a NADH regeneration system was introduced into the *E. coli*, and finally a 154.0 mg/L butanol titer was achieved in a laboratory-scale shake-flask experiment.

Keywords Metabolic engineering · *Escherichia coli* · Butanol · Glycerol · NADH regeneration

Introduction

As the global demand for energy continues to rise, biofuels derived from renewable sources have become increasingly important. Biobutanol has been recognized in recent years as an attractive alternative to traditional fuels. The growing acceptance of biobutanol is largely due to its energy content which is competitive to that of gasoline, potentially allowing for a complete substitution of gasoline without modifications to the existing vehicle engines or fuel delivery infrastructure. However, butanol cannot be synthesized economically using native microorganisms. The industrial fermentation of clostridia for the production of n-butanol is associated with problems such as a spore-forming life cycle, byproducts such as acetone, ethanol, and butyrate, instability during the cultivation, and slow growth (Jones and Woods 1986). Thus, there is interest in producing butanol from a more suitable industrial organism.

Metabolic engineering offers an alternative approach in which synthetic pathways are engineered into user-friendly hosts for the production of these fuel molecules. These hosts could be manipulated to improve the production efficiency. Several investigations describe the successful expression of clostridial butanol pathway genes and butanol synthesis in *Escherichia coli* (Atsumi et al. 2008; Inui et al. 2008; Nielsen et al. 2009), *Pseudomonas putida*, *Bacillus subtilis* (Nielsen et al. 2009), *Saccharomyces cerevisiae* (Steen et al. 2008), and *Lactobacillus brevis* (Berezina et al. 2010). *E. coli* is an industrial platform organism and a convenient host because of the vast amount of information available on its genetic and physiological characteristics and the existing variety of genetic tools to carry out the host modification, but it lacks natural tolerance to butanol. *P. putida* and *B. subtilis* were chosen as heterogonous hosts for butanol synthesis because of their natural tolerance to butanol, 0.75 and 1.25 % (w/v), respectively (Nielsen et al. 2009). A recombinant *S. cerevisiae* butanol-producing strain has recently been constructed (Steen et al. 2008). However, it is impossible for large-scale *S. cerevisiae*-based butanol production to be profitable, not only because of the low productivity of the recombinant strain (2.5 mg/L) but also because of its inability to ferment pentose sugars (Ingram et al. 1999), preventing it from completely utilizing a substrate as cheap and abundant as hydrolyzed biomass. Engineered *L. brevis* could produce butanol, but

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the strain was not able to achieved high concentration, and was not suitable for industrial production (Berezina et al. 2010). Especially using an enzymatic chemical reaction mechanism, butanol can achieved high-level production in *E. coli* (Bond-Watts et al. 2011). Therefore, *E. coli* is still a prospective microorganism host for bio-butanol production

Glycerol is an abundant and inexpensive carbon source due to its generation as a byproduct during biofuel production (Gonzalez et al. 2008, 2010), and it is an appealing substrate for the biological synthesis of butanol. Glycerol metabolism in *E. coli* is very slow, and affects the rate of butanol production. *E. coli* cells absorb glycerol mainly by passive transport. Glycerol is transported to the periplasm through the cell outer membrane channel protein, then through the cell inner membrane porin GlpF to kytoplasm. Then, glycerol turns into dihydroxyacetone phosphate (DHAP) via a series of metabolic reactions, and DHAP is the carbon flow into the EMP pathways (Fig. 1). Therefore, expressing the channel protein gene *GlpF* assisted the glycerol utilization of *E. coli* by enhancing the transport of extracellular glycerol into the kytoplasm (Klaus et al. 1980; Gonzalez et al. 2008).

During the production of butanol,different kinds of byproducts are generated simultaneously both in native clostridia and other engineered strains, and many efforts have been employed to improve the butanol titer by reducing the yield of the byproducts (Atsumi et al. 2008). In the engineered strains, ethanol, succinate, lactate, and acetate have been found to be produced, and these byproducts compete with the acetyl-CoA. Thus, it is practicable to delete such pathways that compete with the butanol pathway.

Recent studies have shown that the transferring pathway requires 5 mol of NADH to produce 1 mol of n-butanol from acetyl-CoA. Two NADH molecules are necessary for the BCD/ETFAB catalyzed reduction of ferredoxin coupled with reduction of crotonyl-CoA (Herrmann et al. 2008; Li et al. 2008), while another three NADH are utilized in the HBD and ADHE2 reactions (Fig. 1). The process of glycerol turning into DHAP in the glycerol metabolism of *E. coli* can produce 2 NADH molecules. However, except for the consumption of NADH by the clostridial butanol pathway, the byproducts pathways also exhaust the NADH. This disturbs the electron balance in the metabolism of the recombinant host and leads to a slow kinetic of the butanol pathway. Although inactivation of *ldhA*, *adhE*, and *frdBC* could reduce the consumption of NADH (Fig. 1), NADH is still a limitomg reason for a low level of butanol production. Therefore, NADH regeneration sounds attractive. Yeast formate dehydrogenase catalyzes the conversion of formate to CO_2 while producing one molecule of NADH (Berríos-Rivera et al. 2002). Due to the formate dehydrogenase of *E. coli* catabolizing formate to CO_2 and H_2 without generation of NADH (Berríos-Rivera et al. 2002), Nielsen et al. (2009) introduced a formate dehydrogenase (*fdh1*) from *S. cerevisiae* and obtained about 74 % butanol production improvement.

This article aims to construct the butanol synthesis pathway of *Clostridium acetobutylicum* into *E. coli* MG1655 (DE3) and to investigate the production of butanol in the recombinant strains. Glycerol was used as the carbon source for butanol production. Therefore, the glycerol transport protein gene *GlpF* was introduced to improve the strain's utilization of glycerol. To reduce byproducts, the synthesis of competing metabolic byproducts was knocked out using λ Red recombination (Datsenko and Wanner 2000). A NADH regeneration system was built in the butanol production strain by introducing the *fdh1* gene from *Candida boidinii* (Berríos-Rivera et al. 2002; Zhang et al. 2009). Consequently, butanol production was gradually improved.

Materials and methods

Strains and growth conditions

Table 1 describes the strains used in this study. *E. coli* MG1655 (DE3) was used as the host strain, and *E. coli* DH5α was used for plasmid amplification in this study. *E. coli* cultures were grown in LB medium, with the addition of ampicillin (100 μg/mL), chloramphenicol (34 μg/mL), and streptomycin (100 μg/mL) as necessary. *Candida boidinii* was purchased from the China Center of Industrial Culture Collection (CICC), and the yeast strain grew in YPD, 1.0 % yeast extract (w/v), 2.0 % peptone (w/v), and 2.0 % glucose (w/v). *Clostridium acetobutylicum* genomic DNA was obtained from the Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Science, Chinese Academy of Science. Strains were kept in 20 % glycerol (v/v) stocks at −80°C.

Plasmid construction

Genes from *Clostridium acetobutylicum* ATCC824 (*thil*, *bcs-operon*, *adhe2*), *E. coli* MG1655 (DE3) (*GlpF*), and *Candida boidinii* (*fdh1*) were obtained by polymerase chain reaction (PCR) using respective genomic DNA templates. The butanol biosynthesis pathway was reconstructed using three compatible expression vectors, pETDuet-1, pACYCDuet-1, and pCDFDuet-1, which possess the *colEI* replicons, p15A replicons, and CDF replicons, respectively. The *thil* was digested with *Bgl*II and *Kpn*I, and ligated into the same sites of pACYCDuet-1 to create pT. Then, the *bcs-operon* was digested with *Nco*I and *Sac*I and ligated into the same sites of pT to create pBT. The *adhe2* was digested with *Nco*I and *BamH*I, and ligated into the same sites of pETDuet-1 to create pA. The *GlpF* was digested with *Bgl*IIand *Xho*I, and ligated

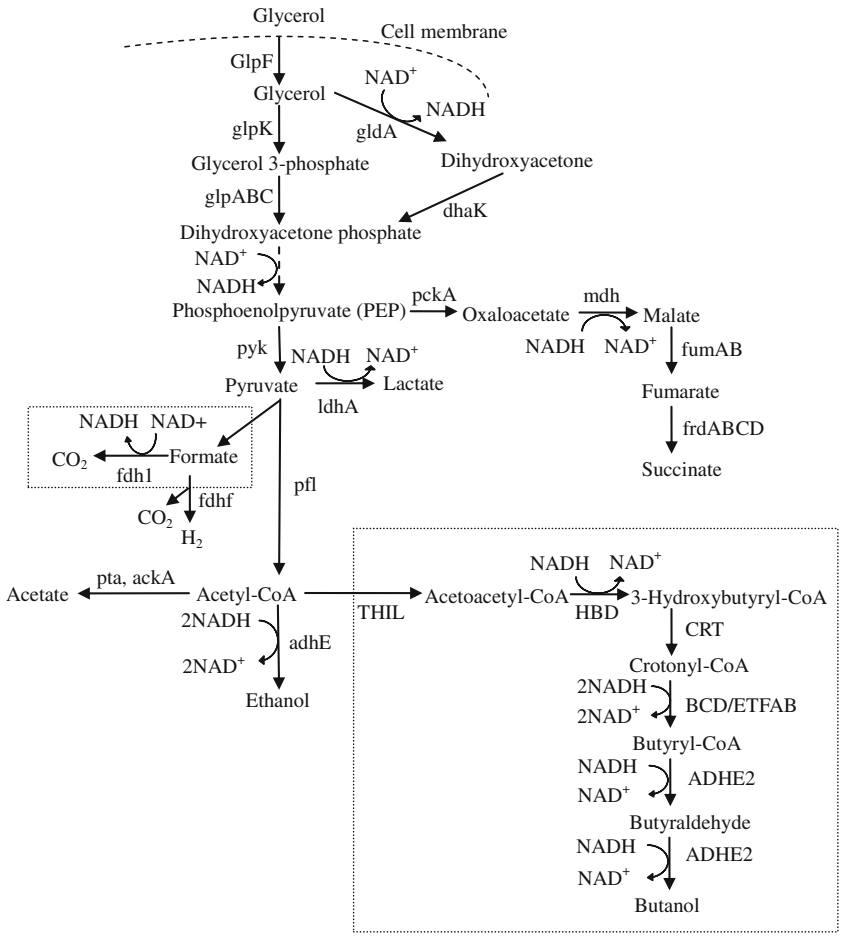

Fig. 1 The anaerobic metabolic pathways of glycerol in *E. coli* and the engineering butanol production metabolic pathway of *E. coli*. The engineered 1-butanol production pathway consists of six enzymatic steps from: *THIL* acetoacetyl-CoA thiolase; *HBD* 3-hydroxybutyryl-CoA dehydrogenase; *CRT* crotonase; *BCD* butyryl-CoA dehydrogenase; *ETFAB* electron transfer flavoprotein; and *ADHE2* aldehyde/alcohol dehydrogenase. Phosphoenolpyruvate and pyruvate were carboxylated to intermediates leading to the byproducts succinate, lactate, and ethanol. *Dashed box* is the newly introduced NAD^+-dependent pathway

into the same sites of pA to create pAG. The *fdh1* was digested with *Bgl*IIand *Xho*I, and ligated into the same sites of pCDFDuet-1 to create pF. Primers for construction of the new plasmids are listed in Table 2.

Table 1 Strains and plasmids used in this study. ("Δ"indicates gene deletion)

Strain or plasmid	Characteristic	Source
DH5a	Host for gene cloning	Our laboratory
MG1655(DE3)	F¯λ¯ilvG-rfb-50 rph-1	Our laboratory
MGΔ3	MG1655 (DE3) ΔadhEΔldhAΔfrdBC	This study
MGΔ4	MG1655 (DE3) ΔadhEΔldhAΔfrdBC Δfdhf	This study
BM	*E. coli* MG1655 (DE3) bearing pA and pBT	This study
BGM	*E. coli* MG1655 (DE3) bearing pAG and pBT	This study
BGMΔ3	*E. coli* MGΔ3 bearing pAG and pBT	This study
FMΔ3	*E. coli* MGΔ3 bearing pF, pAG and pBT	This study
FMΔ4	*E. coli* MGΔ4 bearing pF, pAG and pBT	This study
pACYCDuet-1	Cm, lacI, T7lac	Novagen
pETDuet-1	Ap, lacI, T7lac	Novagen
pCDFDuet-1	Str, lacI, T7lac	Novagen
pBT	pACYCDuet-1 with a DNA fragment containing the *bcs operon* and *thil*	This study
pB	pACYCDuet-1 containing gene *bcs operon*	This study
pA	pETDuet-1 containing gene *adhe2*	This study
pAG	pETDuet-1 containing genes *adhe2* and *GlpF*	This study
pF	pCDFDuet-1 containing gene *fdh1*	This study

Table 2 Primers used in this study

Primer name	Primer sequence (5′ to 3′ direction)
Plasmid construction	
F-thil	CGCGGTACCATGAGAGATGTAGTAATAGT AAGT
R-thil	CGCCTCGAGTTAGTCTCTTTCAACTACGA GCT
F-adhe2	CGGCGCCATGGAAATGAAAGTTACAAATCA
R-adhe2	CCAGGGATCCGCTAATATAATGAAGCAAAG
F-bcs	GAGCCCATGGTCATGGAACTAAACAATGTC
R-bcs	GTCGGAGCTCTGGGGATTCTTGTAAACTTA
F-GlpF	GAAGATCTTCATGAGTCAAACATCAACCTT
R-GlpF	CCCTCGAGGGTTACAGCGAAGCTTTTTGTT
F-fdh1	GGCCGGATCCGATGAAGATCGTTTTAGT CTTAT
R-fdh1	GGCGGAGCTCTTATTTCTTATCGTGTTTAC CGT
Gene inactivation	
F-adhE	ATGGCTGTTACTAATGTCGCTGAACTTAAC GCACTCGTAGAGCGTGTAAAGTGTAGGC TGGAGCTGCTTC
R-adhE	TTAAGCGGATTTTTTCGCTTTTTTCTCAGCT TTAGCCGGAGCAGCTTCTTCATATGAAT ATCCTCCTTAG
F-ldhA	ATGAAACTCGCCGTTTATAGCACAAAACA GTACGACAAGAAGTACCTGCAGTGTAGG CTGGAGCTGCTTC
R-ldhA	TTAAACCAGTTCGTTCGGGCAGGTTTCG CCTTTTTCCAGATTGCTTAAGTCATATGA ATATCCTCCTTAG
F-frdBC	ATGGCTGAGATGAAAAACCTGAAAATTG AGGTGGTGCGCTATAACCCGGAGTGTAG GCTGGAGCTGCTTC
R-frdBC	TTACCAGTACAGGGCAACAAACAGGATTA CGATGGTGGCAACCACAGTTACATATGA ATATCCTCCTTAG
F-fdhf	ATGAAAAAAGTCGTCACGGTTTGCCCCTA TTGCGCATCAGGTTGCAAAATGTGTAG GCTGGAGCTGCTTC
R-fdhf	TTACGCCAGTGCCGCTTCGCGCAGGCGA GTTTTCAACTTGTTGTACTCGTCATATG AATATCCTCCTTAG
Verification	
F-A	CAGTGAGTGTGAGCGCGAGTAA
R-A	GAAGCCGTTATAGTGCCTCAGT
F-I	GGCATGTTTAACCGTTCAGTTG
R-I	CGGCTACTTTCTTCATTGTGGT
F-F	ATAAGGCGGAAGCAGCCAATAA
R-F	GTCAGAACGCTTTGGATTTGGA
F-fdhf	GCCCGTAATATCAGGGAATG
R-fdhf	CGATTGGGTAGGGAGTAA

The inserted genes were verified by plasmid restriction analysis and sequencing. The successful production of the butanol pathway enzymes in *E. coli* was demonstrated by SDS-PAGE and confirmed by enzyme activity detection in the *E. coli* extracts.

Deletion of *E. coli* chromosomal genes

Escherichia coli MG1655 (DE3) genomic DNA genes were deleted by the λ Red recombinase method of chromosomal disruption (Datsenko and Wanner 2000). Transformant MG1655 (DE3), carrying a Red helper plasmid pKD46, was inoculated into LB and grew overnight at 30 °C. The overnight culture was diluted 1/100 in 5 mL LB containing ampicillin, when OD_{600} was up to 0.2, then L-arabinose was added into the medium until the final concentration was 30 mM, and the culture continued to grow for at least 1.5 h until OD_{600} reached about 0.6 at 30 °C with vigorous shaking. After incubation on ice for 20 min, the cells were collected by centrifugation at 4 °C, washed twice with 1 mL ice-cold 10 % glycerol (v/v), and finally resuspended in 50 μL ice-cold 10 % glycerol (v/v). PCR products were gel-purified, digested with *Dpn*I, repurified, and suspended in ddH_2O. Electroporation was carried out with a Bio-Rad Gene Pulser and Pulse Controller (Bio-Rad, USA) using cuvettes (0.2-cm gap) filled with 50 μL electrocompetent cells and 5 μL PCR product, and the following settings: 2.5 kV, 5.5 ms. The electroporated cells were immediately added into 1 mL LB liquid medium, incubated for 1.5 h at 37 °C with 150 rpm shaking, and then spread onto LB agar containing kanamycin at 37 °C. Then, several selected colonies were subjected to PCR to verify the positive colony, The antibiotic resistance cassette was eliminated using a temperature-sensitive helper plasmid, pCP20, To check for gene disruption on the chromosome, PCR analyses were performed using locus-specific primers (ldhA, F-I/R-I; frdBC, F-F/R-F; adhE, F-A/R-A; fdhf, F-fdhf, R-fdhf). A freshly isolated colony was suspended in a PCR working solution. The PCR program was as follows: 94 °C for 6 min, followed by 30 cycles at (1) 94 °C for 1 min, (2) 52 °C for 30 s, and (3) 72 °C for 30 s. Primers for gene inactivation or verification are listed in Table 2.

Enzyme activities

Escherichia coli strains were grown in 50 mL LB liquid medium at 37 °C and 220 rpm. When OD_{600} reached about 0.8, cultures were induced with 1 mM IPTG and grown for an additional 6 h, The cells were collected by centrifugation at 5,000 *g*, 4 °C for 10 min, and resuspended in 5 mL sonication buffer (100 mM Tris–HCl buffer and 2 mM 1,4-dithiothreitol; pH 7.5). The mixture was homogenized

for 15 min with an ultrasonic homogenizer. Cellular debris was removed by centrifugation at 13,000 *g*, 4 °C for 15 min, and the supernatant was used as a cell extract. Protein concentrations were measured using the Bradford method. Thiolase (THIL), β-hydroxybutyryl-CoA dehydrogenase (HBD), crotonase (CRT), and butyryl-CoA dehydrogenase (BCD) activities were assayed at 30 °C in accordance with the published procedures (Inui et al. 2008). For biofunctional butyraldehyde and butanol dehydrogenase (ADHE2) enzyme assay, cultures were grown in 50 mL LB medium in a sealed 50-mL tube at 37 °C in a rotary shaker at 220 rpm. At OD_{600} of 0.8, cultures were induced with 1 mM IPTG and grown for an additional 6 h, cells collected by centrifugation at 5,000 *g*, 4 °C for 10 min, resuspended in 5 mL sonication buffer in 100 mM Tris–HCl buffer (pH 7.0) and homogenized for 15 min with an ultrasonic homogenizer. The crude extracts were then assayed according to the method described earlier (Inui et al. 2008).

Culture medium and cultivation conditions

Strains were streaked from their frozen glycerol stocks onto appropriate LB plates and incubated overnight at 37 °C. Colonies were used to inoculate 5 mL of LB medium in 15-mL tubes overnight at 37 °C at 220 rpm. The 3 mL preinoculum was used to seed 150 mL TB medium supplemented with 5 mg/L glycerol in 250-mL screw-capped flasks. Cultures were first grown under aerobic conditions in shake flasks to promote biomass production. For anaerobic growth, cultures were adjusted to OD_{600} of 1.5 with 40 mL of the same fresh medium with appropriate antibiotics, induced with 1 mM IPTG, and cultures was transferred into 50-mL sealed flasks, and the headspace was evacuated for anaerobic fermentation. In all cases, TB medium was supplemented with appropriate antibiotics. Culture media were shaken at 37 °C for 8–56 h. Samples were centrifuged to pellet cells while the aqueous supernatant was collected for GC and HPLC analysis. The pH and OD_{600} values, for all experiments, were determined from a minimum of 3 independent fermentation flasks.

Metabolite detection

Fermentation samples were centrifuged for 3 min at 13,000 *g* in a microcentrifuge. The supernatant was filtered through a 0.22-μm syringe filter. The produced alcohol compounds were quantified by an Aglient 9860 gas chromatograph (GC) equipped with flame ionization detector (FID) (Agilent, USA). The separation of alcohol compounds was carried out by a SE-54 weakly polar column (Renhua Chromatography Technology Development Center, China). GC oven temperature was initially held at 60 °C for 0.5 min and raised with a gradient of 10 °C/min, then held at 120 °C for 2 min, then raised again with a gradient of 10 °C/min until 220 °C, and finally held for 4 min to clear the column of any remaining chemicals. The injector and detector were maintained at 225 °C. The column was injected with 0.5 μL of the supernatant of culture broth in a split injection mode with a 1:20 split ratio. The internal standard used was isobutanol, and the alcohol content was determined by extrapolation from standard curves using the internal standard to normalize the values. For other metabolites, filtered supernatant was applied to an Agilent 1100 HPLC equipped with an auto-sampler (Agilent) and a BioRad Aminex HPX87 column (BioRad, USA) (0.5 mM H_2SO_4, 0.6 mL/min, column temperature at 35 °C). These metabolites were detected at 210 nm. Concentrations were determined by extrapolation from standard curves.

Results and discussion

Construction of butanol-producing *E. coli*

Butanol synthesis in *E. coli* was investigated via expression of the clostridial genes. MG1655 (DE3) was transformed with pA and pBT (strain BM; see Table 1). Strains were cultured in TB medium supplemented with 0.5 % (w/v) glycerol to assess their ability to synthesize butanol. Following induction with IPTG, butanol was detected in the culture broth; specifically, butanol synthesis after 48 h by strain BM reached up to 18.5 mg/L under anaerobic conditions (Fig. 2). The butanol yield was comparable with earlier efforts to reconstruct the butanol pathway in *E. coli* (Atsumi et al. 2008). In the case of *E. coli* MG1655 (DE3) strain, which was monitored as a control, butanol was not detected in the culture. It is not easy to realize high titers of heterologous 1-butanol production by transferring the clostridial pathway to nonnative hosts, such as *E. coli* (Atsumi et al. 2008; Inui et al. 2008; Nielsen et al. 2009), *P. putida*, *B. subtilis* (Nielsen et al. 2009), *S. cerevisiae* (Steen et al. 2008), and *L. brevis* (Berezina et al. 2010). According to Shen et al. (2011), the clostridial pathway is CoA-dependent, needing a driving force such as the decarbonxylation reaction near the endproducts. However, there is no significant driving force existing in such a pathway to direct the carbon flux through the five reversible steps to butanol.

To improve the utilization of glycerol, we expressed the glycerol transport protein GlpF, and the glycerol consumption was improved 25 % and the butanol production of strain BGM was improved 23 %, reaching 22.7 mg/L after 48 h (Fig. 2). An *E. coli* MG1655 strain was engineered for utilizing crude glycerol to produce ethanol by overexpressing the glycerol dehydrogenase (GldA, encoded by the gene *gldA*) and

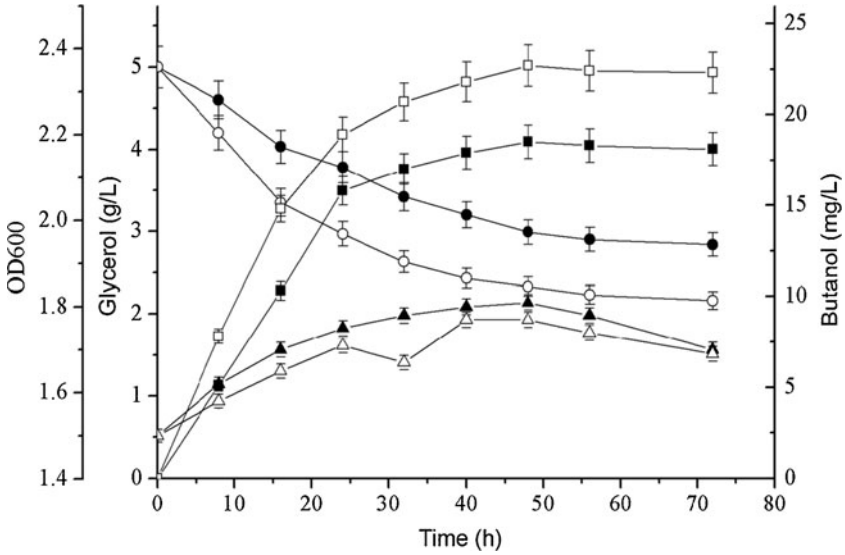

Fig. 2 Consumption of glycerol, butanol accumulation, and growth curves of BM and BGM strains. The results are the mean values of three independent experiments. The *filled symbols* represent BM and the *open symbols* represent BGM. *Squares* butanol, *circles* glycerol, and *triangles* OD600

dihydroxyacetonekinase (DHAK, encoded by the operon *dhaKLM*), since the low activities of GldA and DHAK in wild-type *E. coli* might be a limiting factor in glycerol metabolism. After the overexpressing of the two enzymes, a 3.4-fold increase in the amount of glycerol fermented by 48 h was achieved (Yazdani and Gonzalez 2008). GldA and DHAK are the first two enzymes of the kinetics of glycerol fermentation after the glycerol is transported into the cell (Fig. 1), and normally the bottleneck of glycerol metabolism, so even the glycerol transport was improved. *Clostridium acetobutylicum* only metabolizes glycerol in the presence of glucose, while *Clostridium pasteurianum* can use glycerol as the sole carbon source, but, unfortunately, *Clostridium pasteurianum* produces a mixture of butanol, 1,3-propanediol and ethanol (Biebl 2001; Taconi et al. 2009). Therefore, the engineered *E. coli* is competitive in butanol production.

Enzyme assays for butanol synthetic enzymes expressed in *E. coli*

Cell extracts obtained from *E. coli* MG1655 (DE3) and BM were analyzed for THIL, HBD, CRT, and ADHE2 enzyme activities. The THIL, HBD, and CRT activities in *E. coli* MG1655 (DE3) and BM were measured spectrophotometrically, and the results showed that the THIL, HBD, and CRT activities of BM were, respectively, 3, 16, and 138 times greater than those of the control strain. The THIL specific activity of the BM strain, calculated based on the decrease in acetoacetyl-CoA, was 0.339 U/mg protein, which was higher than that of the control strain, 0.11 U/mg protein (Table 3). The THIL specific activity of control strain MG1655 (DE3) was higher than results of earlier researches, such as 0.043 U/mg protein of the wild strain JM109 (Inui et al. 2008), and undetected THIL activity of the wild strain XL1-Blue (Berezina et al. 2010). THIL was the first key enzyme in the process of butanol production whose higher activity expressed in recombinant strain could lead to a high yield of butanol. However, the enhancement of THIL activity in the present strain was lower than that of the two enzymes catalyzing the following two reactions in the butanol synthesis pathway, becoming the bottleneck of butanol production. The specific activity of HBD in strain BM, determined from the decrease in NADH, was 0.344 U/mg protein, yet the specific activity of the control strain was 0.022 U/mg protein (Table 3). The specific activity of CRT was calculated by the decrease in crotonyl-CoA, which was 1.38 U/mg protein, while the specific activity of the control stain was 0.010 U/mg protein. Thus, the HBD and CRT activity were greatly improved in the recombinant strain. We were unable to detect BCD activity in the recombinant strain and the control strain, nevertheless, the functional expression of butanol pathway was confirmed by butanol production. Recombinant butyraldehyde and butanol dehydrogenase activities were 0.117 and 0.078 U/mg protein, respectively, and were comparable with earlier researches (Inui et al. 2008; Berezina et al. 2010).

Table 3 Enzyme activities of *E. coli* MG1655 (DE3) and BM strains

Strain	Enzyme activity (U/mg protein)			
	THIL	HBD	CRT	ADHE2
BM	0.339	0.344	1.38	0.117
MG1655(DE3)	0.110	0.022	0.010	0.078

Data represent the mean values calculated from experiments in triplicate. One unit of activity was defined as 1 μmol of substrate utilized or product formed per minute

Effects of eliminating by-product pathways on butanol production

Anaerobic conditions result in the majority of the glycerol being converted to ethanol, succinate, and lactate (Fig. 3), with butanol representing only a minor product in wild-type *E. coli*. Eliminating the pathways to the undesired metabolites ethanol, succinate, and lactate is a feasible strategy to improve butanol titers and yields from glycerol. The key enzyme genes *adhE*, *frdBC* and *ldhA* of MG1655 (DE3) for ethanol, succinate, and lactate synthesis were successfully deleted using λ Red recombination (Fig. 4a, b). The majority of byproducts of the strain BGM were composed of succinate, lactate, and ethanol, which were at concentrations of 3,800, 350, and 1,700 mg/L, respectively. Titers of the three byproducts in the fermentation broth decreased to 2,500, 163, and 740 mg/L, respectively, after the three genes were deleted (Fig. 3). Meanwhile, eliminating byproduct pathways resulted in an increase in butanol production, which for strain BGMΔ3 achieved 97.9 mg/L (Fig. 5), which was an improvement of about 331 %. Analogously, the butanol yield was doubled when the same three genes of *E. coli* BW25113 were deleted (Atsumi et al. 2008), which demonstrated that an increase in butanol production was available by deleting the three byproducts pathway genes. However, we found the acetate concentration in the fermentation broth was enhanced by 85 % (data not shown). Accordingly, the glycolytic flux was intended for native products (Nielsen et al. 2009). Although some of the pathways for native byproducts formation were reduced, according to the mass balance, the glycolytic flux to the rest of the byproducts as well as to the butanol was raised. In addition, the re-engineering of pathways often leads to imbalanced gene expression, creating a bottleneck in the biosynthetic pathway that reduces the production of the target compound. The clostridial butanol synthesis pathway utilizes both NADH and reduced ferredoxin (Bcd-EtfAB complex) as sources of reducing power (Li et al. 2008). The NADH driving force could be established by deleting the mixed acid fermentation reactions (ethanol, lactate, and succinate). However, this strategy resulted in the reduction of the abilities of grow anaerobically and of NADH recycling (Shen et al. 2011). Therefore, to create a NADH recycling pathway will be advantageous for butanol synthesis.

Increase of NADH availability by expressing an NAD^+-dependent formate dehydrogenase

In an effort to improve butanol synthesis, we explored the effects of introducing formate dehydrogenase (*fdh1*) from *Candida boidinii*. Yeast formate dehydrogenase catalyzes the conversion of formate to CO_2 while producing one molecule of NADH. While formate dehydrogenase exists in *E. coli*, the bacterial enzyme catabolizes formate to CO_2 and H_2 without generation of NADH. For this reason, yeast formate dehydrogenase has been exploited in a variety of biocatalytic applications requiring enough NADH molecules for product formation (Berríos-Rivera et al. 2002; Harris et al. 2000; Sanchez et al. 2005). Expression of *fdh1* in strain BGMΔ3 resulted in the generation of strain FMΔ3, as shown in Table 1, and the bio-butanol synthesis of strain FMΔ3 was 154.0 mg/L after 48 h fermentation (Fig. 5), which was about a 57 % butanol production improvement over strain BGMΔ3. Expression of FDH from *Candida boidinii* was demonstrated an efficient way to increase the intracellular NADH driving force (Shen et al. 2011). However, the butanol yield of FMΔ3 was about 8.6 % of the theoretic yield (maximum theoretic yield is 0.6 g/g of glycerol), which indicated that the butanol yield could be further improved by additional manipulation in the pathway or a certain reaction.

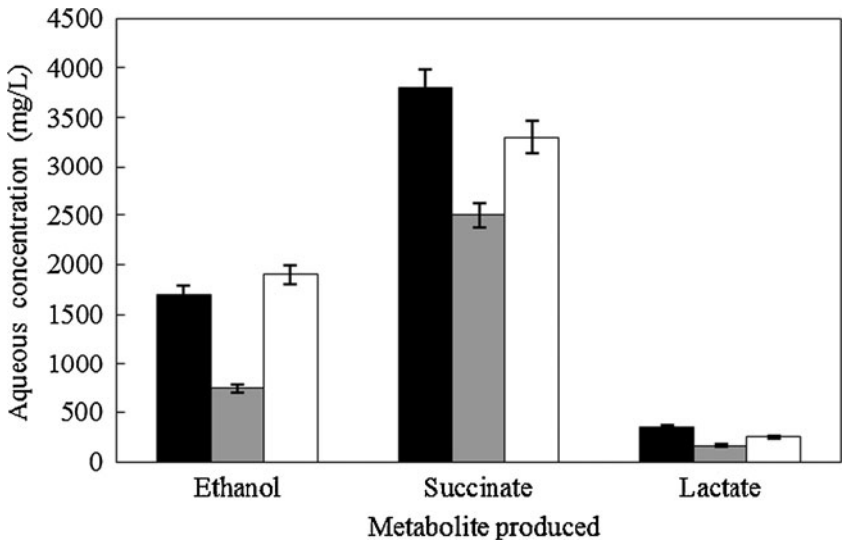

Fig. 3 Byproduct formation with the butanol production by strains BGM (*black*), BGMΔ3 (*gray*), FMΔ3 (*white*)

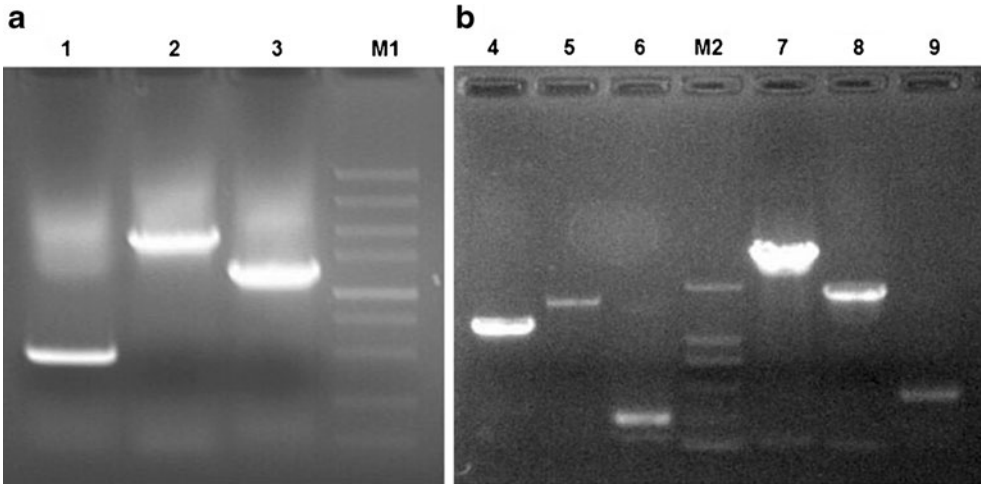

Fig. 4 **a** PCR verification of the ldhA mutant in MG1655 (DE3). *1* positive clone without kanamycin resistance; *2* recombinant with kanamycin resistance; *3* wild-type; *M1* DL5000. **b** PCR verification of the frdBC adhE mutants. *4* wild-type; *5* recombinant with kanamycin resistance; *6* positive clone without kanamycin resistance; *M2* DL2000; *7* wild-type; *8* recombinant with kanamycin resistance; *9* positive clone without kanamycin resistance

Characterization of strain FMΔ3 fermentation byproducts revealed that succinate, lactate, and ethanol, which were the endproducts of NADH-consuming pathways, were the main byproducts after 48 h fermentation, and their titers were improved compared with the strain BGMΔ3 (Fig. 3). Expression of *fdh1* in BGMΔ3 not only resulted in butanol production increase but also increased the flux of each natural NADH-consuming pathway of *E. coli*. Overall, the elevation of fermentative byproducts reflects an increase in the intracellular NADH/NAD^+ ratio (de Graef et al. 1999). We also further explored the influence of the *E. coli* native formate dehydrogenase on butanol production by deleting *fdhf* of the strain FMΔ3 to construct the strain FMΔ4. However, the strain FMΔ4 from which the gene *fdhf* was deleted produced alower growth rate and 22.6 mg/L butanol yield (Fig. 5).

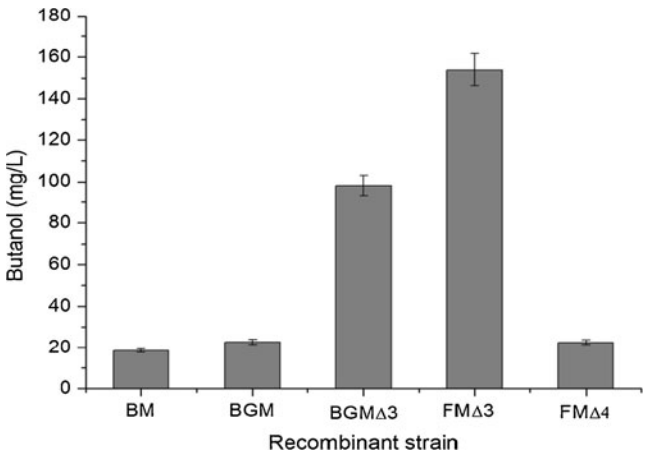

Fig. 5 Butanol production of engineered strains. These strains were grown in an anaerobic condition at 37 °C for 48 h in TB medium in shake flasks

Conclusion

The bacterium *E. coli* is a well-characterized microorganism from both the genetic and metabolic perspectives, and there is a vast availability of genetic tools for its engineering. In this article, we engineered *E. coli* for the anaerobic production of butanol from glycerol in TB medium. To achieve this, we introduced *thil*, *adhe2*, and *bcs-operon* from *Clostridium acetobutylicum* into *E. coli* MG1655 (DE3) using two plasmids, to construct the recombinant *E. coli* (BM) strain, and the butanol titer could achieve 19.0 mg/L after 48 h fermentation. To improve glycerol utilization of the BM strain, we expressed the glycerol transport protein GlpF, and butanol production of the strain BMG was improved by 23 %. In BM and BMG culture broth, ethanol and organic acids were found as the main byproducts. Therefore, further modification was carried out by disrupting the *adhE*, *ldhA*, and *frdBC* genes responsible for the byproduct synthesis, to increase the butanol yield. And the butanol yield of the strain BGMΔ3 achieved more than 3 times that of the BGM strain. Moreover, a NADH regeneration system was introduced into the strain BGMΔ3 by taking advantage of the *fdh1* from yeast *Candida boidinii*, which resulted in a further improvement of the butanol titer up to 154.0 mg/L in a shake flask culture.

We have shown the feasibility of the recombinant *E. coli* producing butanol from glycerol; however, the final butanol yield was not high compared with some earlier studies. The lower activity THIL of the clostridial butanol synthesis pathway and native GldA and DHAK in *E. coli* might be responsible for the low titer of butanol. Shen et al. (2011) proposed that the acetyl-CoA driving force is also as significant as the NADH driving force in the efficient production of the nonnative products. By using the NADH and acetyl-CoA driving forces, coupled with the irreversible reaction catalyzed by trans-enoyl-CoA reductase (Ter), we achieved both high titer (30 g/L) and high yield (70–88 % of the theoretical).

Accordingly, in subsequent research, the optimization of the glycerol conversion rate, *thil* expression, and acetyl-CoA driving force, will be our key focus.

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