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ORIGINAL PAPER

# Engineering a homobutanol fermentation pathway in *Escherichia coli* EG03

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**Abstract** A homobutanol fermentation pathway was engineered in a derivative of *Escherichia coli* B (glucose [glycolysis] => 2 pyruvate + 2 NADH; pyruvate [pyruvate dehydrogenase] => acetyl-CoA + NADH; 2 acetyl-CoA [butanol pathway enzymes] + 4 NADH => butanol; summary stoichiometry: glucose => butanol). Initially, the native fermentation pathways were eliminated from *E. coli* B by deleting the genes encoding for lactate dehydrogenase (*ldhA*), acetate kinase (*ackA*), fumarate reductase (*frdABCD*), pyruvate formate lyase (*pflB*), and alcohol dehydrogenase (*adhE*), and the pyruvate dehydrogenase complex (*aceEF-lpd*) was anaerobically expressed through promoter replacement. The resulting strain, *E. coli* EG03 (Δ*frdABCD* Δ*ldhA* Δ*ackA* Δ*pflB* Δ *adhE* Δ*pdhR ::pflBp6-aceEF-lpd* Δ*mgsA*), could generate 4 NADH for every glucose oxidized to two acetyl-CoA through glycolysis and the pyruvate dehydrogenase complex. However, EG03 lost its ability for anaerobic growth due to the lack of NADH oxidation pathways. When the butanol pathway genes that encode for acetyl-CoA acetyltransferase (*thiL*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*, *etfA*, *etfB*), and butyraldehyde dehydrogenase (*adheII*) were cloned from *Clostridium acetobutylicum* ATCC 824, and expressed in *E. coli* EG03, a balanced NADH oxidation pathway was established for homobutanol fermentation (glucose => 4 NADH + 2 acetyl-CoA => butanol). This strain was able to convert glucose to butanol (1,254 mg $l^{-1}$) under anaerobic condition.

**Keywords** Butanol fermentation · Biofuel · *E. coli* · Reducing power · NADH · Metabolic engineering

## Introduction

Butanol is widely used as a solvent, plasticizer, and flavoring agent in the food and beverage industry. The estimated worldwide consumption is ~1.3 billion liters per year, with 830 million liters consumed by the United States [24]. Due to its high energy content, butanol is considered to be a superior transportation biofuel that can be produced from renewable resources. In this regard, butanol production needs to be increased dramatically because the demand for transportation fuel is over 1.5 billion liters per day for the U.S. alone [7].

Butanol can be produced via fermentation by several species of *Clostridium,* such as *C. acetobutylicum*. However, as a result of high production costs, clostridial butanol fermentation was outcompeted by the petrochemical industry in the 1960s [12]. Nevertheless, in recent years, interest has grown for economic production of butanol from cellulosic biomass via microbial fermentation due to the increased need of switching to renewable and environmentally friendly fuel sources. The traditional clostridial fermentation, however, produces a mixture of acetone, butanol, and ethanol (known as ABE fermentation). Acetone is considered to be an undesirable fuel component due to its corrosiveness to plastic and rubber engine components. Therefore, minimizing acetone

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production is needed to increase the butanol yield, simplify product recovery processes, and improve the fuel quality of butanol fermentation [16].

Manipulations of fermentation conditions and/or using metabolic engineering approaches have been explored to minimize acetone production in clostridial fermentation [11, 14, 25, 27]. These efforts, however, often resulted in negative impacts on butanol production due to the intrinsic feature of its biphasic fermentation pathways, and/or lack of control over sporulation, which eventually halts butanol production. Recently, an isopropanol pathway was developed in *C. acetobutylicum* ATCC 824 by cloning a primary/secondary alcohol dehydrogenase gene (*sadh*) from *C. beijerinckii* NRRL B-593, which converts acetone to isopropanol [14]. This resulted in IBE fermentation (isopropanol, butanol, and ethanol), which is more desirable since isopropanol can be used as a fuel additive. Although acetone production was eliminated in IBE fermentation, the slow growth rate, spore-forming lifestyle, and biphasic feature of clostridial fermentation remains a challenge for industrial fermentation.

An alternative to clostridial fermentation is to clone the butanol pathway genes into *Escherichia coli*, which has been previously shown to be a suitable host for production of bioethanol [17]. In prior efforts, although butanol was produced, a homobutanol pathway has not been established due to the limited NADH-reducing power output in *E. coli* [1, 9]. Specifically, in butanol pathway, one butanol produced from two acetyl-CoA requires the input of 4 NADH [16, 23], while the *E. coli* host can only provide 2 NADH when glucose is oxidized into two acetyl-CoA through glycolysis and pyruvate-formate lyase under anaerobic condition [5]. Deleting competing fermentation pathways improved butanol production by eliminating the competition for reducing power; however, the maximum reducing power output of 2 NADH remained the same and was unable to meet the 4 NADH requirement of homobutanol fermentation.

In this study, we report cloning the butanol pathway genes from *C. acetobutylicum* into a derivative of *E. coli* B to establish a homobutanol pathway. The butanol pathway genes were cloned into a pUC19 based plasmid (pEAG13), which was then transformed into an engineered derivative of *E. coli* B that has all competing fermentation pathways removed and anaerobically expressed pyruvate dehydrogenase complex (*E. coli* EG03), which could generate additional NADH needed for the homobutanol pathway (glucose [glycolysis] => 2 pyruvate + 2 NADH; pyruvate [pyruvate dehydrogenase complex] => acetyl-CoA + NADH; 2 acetyl-CoA [butanol pathway enzymes] + 4 NADH => butanol; summary stoichiometry: glucose => butanol). *E. coli* EG03 (pEAG13) was able to produce 1,254 mg $l^{-1}$ of butanol from glucose under anaerobic conditions.

## Materials and methods

### Bacterial strains, plasmids, primers, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Bacterial cultures were grown in Luria–Bertani (LB) broth (g $l^{-1}$: tryptone 10, yeast extract 5, and NaCl 5) or on LB plates (agar 15 g $l^{-1}$) [22]. During plasmid/strain construction, antibiotics were added to the media accordingly: kanamycin, 50 μg $ml^{-1}$; ampicillin, 50 μg $ml^{-1}$. Once the bacterial strain was transformed with the plasmid containing the butanol pathway genes, it was grown on LB containing 125 μg $ml^{-1}$ ampicillin to prevent plasmid curing. For anaerobic cell growth and fermentation, the cultures were supplemented with 50 g $l^{-1}$ glucose, and were grown in 8.5 ml of media in 9-ml screw-cap tubes, which became anaerobic as the small amount of oxygen was consumed by the cells.

### DNA techniques

Standard methods were used for plasmid construction, transformation, and electroporation [18, 22]. Plasmid DNA was isolated by using a QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Gel extractions were performed using a QIAquick Gel Extraction Kit (QIAGEN). DNA was purified by either ethanol precipitation [2] or by using a Wizard Plus Minipreps DNA Purification Systems Kit (Promega). PCR methods were performed as previously described [8]. Briefly, the PCR reactions were performed in 50 μl volumes containing 50 ng of *C. acetobutylicum* 824 genomic DNA, 300 pmol of each oligonucleotide primer (Table 1), 25 μl of QIAGEN Taq PCR Master Mix (~3.5 units of Taq DNA polymerase). The PCR amplification was carried out as follows: initial denaturation at 94 °C for 3 min, 35 amplification cycles, each consisting of four steps: denaturation at 94 °C for 1 min, annealing at 50 °C for the five gene operon (*crt-bcd-etfA-etfB-hbd*) (or annealing at 60 °C for the *thil* and *adheII* genes) for 1 min, extension at 60 °C for 6 min for the five gene operon (or at 72 °C for 3 min for the *thil* and *adheII* genes).

The chromosomal gene deletion was performed following previously described procedures [6, 19, 29]. Briefly, PCR primers that were partially complementary to the target gene and the antibiotic cassette (FRT-*kan*-FRT) of pKD4 were used to amplify the cassette. This antibiotic cassette contains the kanamycin resistant gene flanked by repeated flipase recognition target (FRT) sites [6]. After purification, the amplified DNA was electroporated into *E. coli* SZ470 (pKD46) by a Micropulser (Bio-Rad). Kanamycin resistant colonies were selected for potential target gene replacement by the FRT-*kan*-FRT cassette via

**Table 1** Strains, plasmids, and primers used in this study

| *E. coli* strains | Relevant characteristics | Sources |
|---|---|---|
| DH5α | Δ*lac*ZM15 *recA* | Invitrogen |
| B | Wild-type | ATCC11303 |
| SZ470 | Δ*frdABCD* Δ*ldhA* Δ*ackA* Δ*pflB* Δ*pdhR::pflBp6-aceEF-lpd* Δ*mgsA* | [28] |
| EG03 | SZ470, Δ*adhE* | This study |
| Plasmids | | |
| pKD4 | *FRT-kan-FRT* cassette | [6] |
| pKD46 | *bla*, *γ β exo* (red recombinase), temperature-conditional replicon | [6] |
| pFT-A | *bla*, *flp*, temperature-conditional replicon | [19] |
| pCR2.1 | *bla*, *kan*, TA cloning vector | Invitrogen |
| pUC19 | *bla* cloning vector | New England Biolabs |
| pEAG01 | 1.9-kb fragment containing *C. acetobutylicum thiL* gene cloned into pCR2.1 | This study |
| pEAG07 | 3.1-kb fragment containing *C. acetobutylicum adheII* gene cloned into pCR2.1 | This study |
| pEAG09 | 5.3-kb fragment containing *C. acetobutylicum crt-bcd-etfA-etfB-hbd* operon cloned into pCR2.1 | This study |
| pEAG13 | 1.9-, 3.1-, and 5.3-kb fragments containing *thiL*, adheII *and crt-bcd-etfA-etfB-hbd* operon were cloned from pEAG01, pEAG07 and pEAG09 into pUC19 | This study |
| Primers | | |
| *thiL* primer 1 | GGAGA**GCATGC**CCATATGGTGATGGAAAGGCTTCAGA | This study |
| *thiL* primer 2 | GGAGA**CCCGGG**GGAGA**GGCGCGCC**CGCCTAGTACTGCATTAGCCTCA | This study |
| *crt-bcd-etfA-etfB-hbd* primer 1 | GGAGA**GGCGCGCC**TGTTGAGGTTCGGGACGTAG | This study |
| *crt-bcd-etfA-etfB-hbd* primer 2 | GGAGA**CCCGGG**GGAGA**GCGGCCGC**CCATATTATAATCCCTCCTC | This study |
| *adheII* primer 1 | GGAGA**GCATGC**GGAGA**GCGGCCGC**CTAATAATACGTAATACACCCACTTAT | This study |
| *adheII* primer 2 | GGAGA**CCCGGG**GGATACAAGGTAATTAATATTAAAGATGGTACA | This study |

The bold indicates the added restriction enzyme site

double homologous recombination. To verify the gene deletion, PCR was performed and the product size was analyzed. The kanamycin antibiotic marker was then removed from the chromosome via site-specific recombination by flipase (FLP recombinase) using a temperature-conditional helper plasmid known as pFT-A [19].

## Butanol production

*Escherichia coli* EG03 (pEAG13) and the control strain were grown in a test tube containing 10 ml of LB broth supplemented with glucose (50 g $l^{-1}$) and ampicillin (125 μg $ml^{-1}$). The culture was grown in a rotator (200 rpm) for 24 h at 30 °C. This 24-h culture (10 ml) was used to inoculate a 2-l flask containing 500 ml of LB broth supplemented with glucose (50 g $l^{-1}$) and ampicillin (125 μg $ml^{-1}$). The flask culture was grown with shaking at 200 rpm for 24 h at 30 °C. The culture was centrifuged at 4 °C, 5,000 rpm (~3,015 × *g*) for 10 min, and the collected cell pellet was resuspended in 50 ml of minimal media (per liter: 3.5 g of $KH_2PO_4$; 5.0 g of $K_2HPO_4$; 3.5 g of $(NH_4)_2HPO_4$, 0.25 g of $MgSO_4{\cdot}7H_2O$, 15 mg $CaCl_2{\cdot}2H_2O$, 0.5 mg of thiamine, and 1 ml of trace metal stock) containing glucose (50 g $l^{-1}$) and ampicillin (125 μg $ml^{-1}$). The trace metal stock was made as described by Causey et al. [3]. The culture was grown in a sealed serum bottle with stirring in an anaerobic jar at 30 °C for 60 h. Supernatant from this culture was used to determine the amount of butanol produced.

## Analyses

Cell growth was estimated by optical density at 550 nm using a spectrophotometer (Unico 1100). Sugar and organic acid concentrations of fermentation samples were analyzed by HPLC (Waters equipped with a refractive index detector). Butanol (and ethanol) concentrations were determined by gas chromatography (GC) (Varian CP3800 equipped with a flame ionization detector and a capillary column). A Bio-Rad HPX 87H column with 4 mM $H_2SO_4$ as the mobile phase (10-μl injection, 0.4 ml $min^{-1}$, 45 °C) was used for HPLC analysis [29]. For GC analysis, 50 mM 1-propanol was used as an internal standard. GC samples were prepared by mixing 890 μl $diH_2O$, 100 μl of culture supernatant, and 10 μl of 1-propanol internal standard. A total of 1 μl of the prepared sample was then injected for GC analysis [30].

## Results

### Engineering an *E. coli* B platform with a doubled reducing power output (4 NADH)

In *E. coli*, two NADH are normally generated when glucose is oxidized into pyruvate during glycolysis. There is no additional NADH production when pyruvate is oxidized into acetyl-CoA (and formate) by pyruvate formate lyase under anaerobic condition. The reducing power present in formate is dissipated into $H_2$ when formate is converted into $H_2$ and $CO_2$ by the formate hydrogenase complex (FHL) [5]. Two NADH will be the maximum reducing power output when glucose (or equivalent) is oxidized into two acetyl-CoA. This presents a reducing power challenge for establishing a homobutanol pathway in *E. coli* even with elimination of the competing NADH oxidation pathways because the clostridial butanol pathway requires 4 NADH input for conversion of two acetyl-CoA into one butanol. To this end, increasing NADH output of glucose catabolism is needed to meet the reducing power requirement of homobutanol fermentation in *E. coli*.

In our previous study, a native homoethanol pathway was engineered in *E. coli* B by eliminating the competing fermentation pathways and anaerobically expressing the pyruvate dehydrogenase complex (PDH) [4, 28, 29]. Since one NADH is generated when pyruvate is oxidized into acetyl-CoA by PDH, there is a total of 4 NADH output when glucose is oxidized into two acetyl-CoA, which are further converted into two ethanol by alcohol dehydrogenase (AdhE), achieving a balanced redox of anaerobic homoethanol fermentation (glucose [glycolysis] => 2 pyruvate + 2 NADH; pyruvate [pyruvate dehydrogenase complex] => acetyl-CoA + NADH; acetyl-CoA [alcohol dehydrogenase] + 2 NADH => ethanol; summary stoichiometry: glucose => 2 ethanol). This homoethanol strain, SZ470, was an appropriate starting strain for the development of a homobutanol pathway with a balanced reducing power. To achieve this goal, the native *adhE* gene, which encodes for alcohol dehydrogenase, was deleted from SZ470 via double homologous recombination. The gene knockout was confirmed by performing PCR of the deleted region of DNA. The resulting strain had no remaining fermentation pathways and was named *E. coli* EG03 (Fig. 1). As expected, EG03 lost its ability to grow anaerobically due to the lack of NADH oxidation pathways. However, there is a 4 NADH output when glucose is oxidized into two acetyl-CoA via glycolysis and the pyruvate dehydrogenase complex when an NADH oxidation pathway is established by the clostridial butanol pathway.

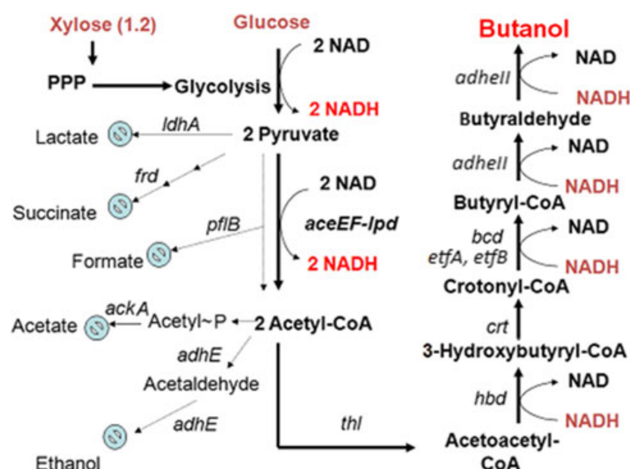


**Fig. 1** Homobutanol fermentation pathway in EG03. The homobutanol fermentation pathway that was engineered in EG03, along with the deleted competing fermentation pathways, are shown. The *stop symbol* represents the pathway was blocked via gene deletion. The deleted genes encode for lactate dehydrogenase (*ldhA*), acetate kinase (*ackA*), fumarate reductase (*frdABCD*), pyruvate formate lyase (*pflB*), and alcohol dehydrogenase (*adhE*). The pyruvate dehydrogenase complex (*aceEF-lpd*) was anaerobically expressed through promoter replacement

### Cloning butanol pathway genes from *C. acetobutylicum* ATCC 824

The clostridial butanol pathway involves the conversion of two acetyl-CoA into one butanol via six biochemical steps, catalyzed by at least five enzymes [16]. In addition, it is reported that three enzymes, alcohol dehydrogenase I (AdheI), alcohol dehydrogenase II (AdheII), and butanol dehydrogenase (BdhAB), play a role in the last step (converting butyryl-CoA into butanol). Of those three enzymes, however, AdheII has a higher affinity for butyryl-CoA than for acetyl-CoA, favoring butanol production [9]. It has also been reported that AdheII is favorable over the native *E. coli* AdhE enzyme since it has a lower affinity for acetyl-CoA, which results in lower ethanol yields [1]. Furthermore, five genes are required for the conversion of acetoacetyl-CoA to butyryl-CoA (crotonase (Crt), butyryl-CoA dehydrogenase (Bcd), electron transfer flavoprotein (etfAB), and 3-hydroxybutyryl-CoA dehydrogenase (Hbd)), which are located in a single operon (*crt-bcd-etfA-etfB-hbd*) in Clostridium. To facilitate the cloning process, the butanol pathway genes were cloned in three DNA fragments containing *thiL*, *crt-bcd-etfA-etfB-hbd*, and *adheII* genes, and were inserted into a pCR2.1 TOPO cloning vector, resulting in plasmids pEAG01, pEAG09, and pEAG07, respectively. The butanol pathway genes were removed by digesting them with the corresponding restriction enzymes that flank the genes in pEAG01 (*SphI/XmaI*), pEAG09 (*AscI/XmaI*), and pEAG07 (*SphI/XmaI*),

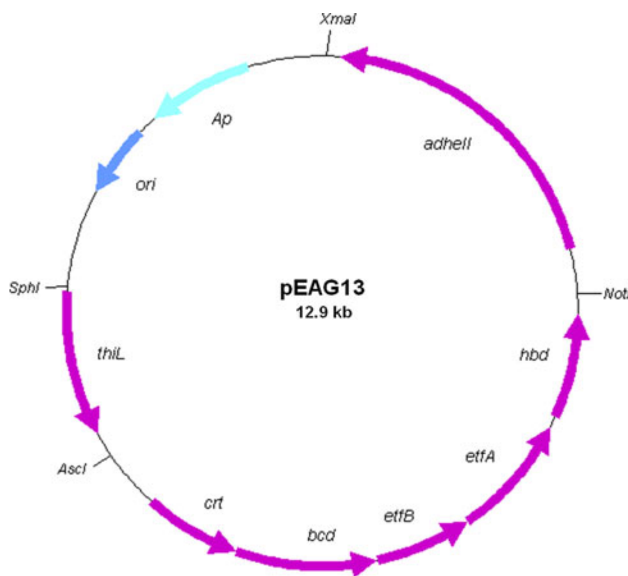


**Fig. 2** The completed butanol pathway plasmid, pEAG13. The vector is pUC19, which contains an ampicillin resistance gene (*bla*). The location of each gene and direction of transcription is indicated by *arrows*. The restriction enzyme cut sites that were used to insert the genes are also shown. The butanol pathway enzymes are acetyl-CoA acetyltransferase (*thiL*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*, *etfA*, *etfB*), and butyraldehyde dehydrogenase (*adheII*)

and were then ligated into pUC19, resulting in the plasmid pEAG13. pEAG13 contains a complete butanol pathway gene (*thiL* + *crt-bcd-etfA-etfB-hbd* + *adheII*) (Fig. 2). It is worthy to note that these genes contain their native clostridial promoters and ribosomal binding sites.

### Butanol production

Transforming plasmid pEAG13 into *E. coli* EG03 should theoretically establish a homobutanol pathway with balanced reducing power, and enable *E. coli* EG03 (pEAG13) to grow anaerobically provided that all butanol pathway genes are expressed efficiently (Fig. 1). Initial testing showed that *E. coli* EG03 (pEAG13) was indeed able to grow in anaerobic screw-cap tubes ($\sim OD_{550}$ of 0.5). However, butanol is produced in negligible amounts. In subsequent tests, concentrated cell cultures were prepared in order to produce a measurable amount of butanol under anaerobic conditions. Initially, an overnight culture (10 ml) of *E. coli* EG03 (pEAG13) and the control strain was used to inoculate a shaking flask. The culture was centrifuged, and the collected cells were transferred to a serum bottle containing minimal salts media, glucose, and ampicillin. The cells of *E. coli* EG03 (pEAG13) and the control strain ($\sim OD_{550}$ of 32) were then incubated with stirring in an anaerobic jar for 60 h. The resulting culture was pelleted, and the supernatant was analyzed for butanol production by gas chromatography (GC). As shown in Fig. 3, *E. coli* EG03 (pEAG13) produced 1,254 mg $l^{-1}$ butanol and 2,072 mg $l^{-1}$ ethanol, while the control strain *E. coli* EG03 produced 1,637 mg $l^{-1}$ ethanol and no butanol. These results demonstrated that the butanol pathway was indeed established in *E. coli* EG03 (pEAG13) with balanced reducing power under anaerobic conditions.

Based on HPLC analysis, no significant amounts of metabolites, other than butanol and ethanol, were produced by *E. coli* EG03 (pEAG13). According to the following stoichiometry equations [(1) and (2)] for the butanol and ethanol (*mhpF*- *adhP* or *adheII*) production pathways present in *E. coli* EG03 (pEAG13), the amount of glucose consumed (8,100 mg $l^{-1}$), the amount of butanol (1,254 mg $l^{-1}$) and ethanol (2,072 mg $l^{-1}$) produced, and the estimated amount of $CO_2$ released (3,383 mg $l^{-1}$) from Eqs. (1) and (2), the calculated carbon recovery would be ~87 %. We assumed that some of the unaccountable 13 % carbon might be used for cell growth, although there was not a significant increase in the $OD_{550}$ detected (measured) during the 60-h concentrated cell fermentation.

$$C_6H_{12}O_6 \rightarrow C_4H_9OH\,(\text{butanol}) + 2CO_2 + H_2O \qquad (1)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH\,(\text{ethanol}) + 2CO_2 \qquad (2)$$

The significant amount of ethanol produced in the control strain was unexpected since the native *adhE* gene was deleted. In *E. coli*, however, there is a potential ethanol pathway present that is encoded by two genes, *mhpF* and *adhP*. The *mhpF* gene encodes for acetaldehyde dehydrogenase 2, which may convert acetyl-CoA to acetaldehyde, and *adhP* encodes for alcohol dehydrogenase, which may convert acetaldehyde to ethanol. This pathway might be responsible for the ethanol production in *E. coli* EG03. To increase the butanol yield, however, this potential ethanol pathway should be eliminated in the future. Nevertheless, there was a 21 % increase in ethanol production when the butanol pathway genes were transformed into *E. coli* EG03. This increase in ethanol production could be attributed to the *adheII* gene of the butanol pathway. AdheII is a bifunctional enzyme that can convert butyryl-CoA to butyraldehyde and butyraldehyde into butanol, as well as convert acetyl-CoA into acetaldehyde and acetaldehyde into ethanol.

## Discussion

The obligate anaerobic requirement, slow growth rate, spore-forming lifestyle, and biphasic feature of clostridial fermentation, continue to be a challenge for industrial butanol fermentation [10]. Attempts have been made to engineer butanol-producing strains in alternative hosts. Steen reported that an engineered strain of *Saccharomyces cerevisiae* produced 2.5 mg $l^{-1}$ of butanol [26]. While this

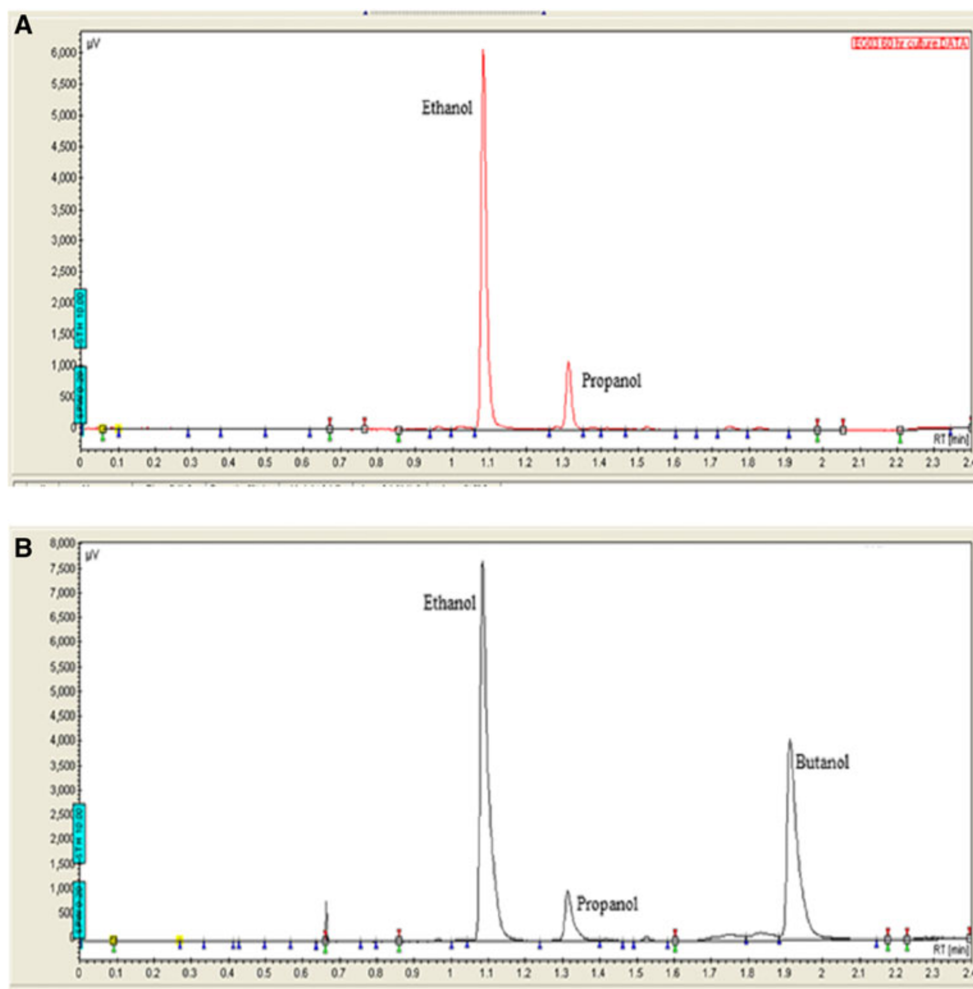


**Fig. 3** Butanol production. **a** *E. coli* EG03 control. **b** Butanol strain *E. coli* EG03 (pEAG13). 1-proponol was used as an internal standard

strain of *S. cerevisiae* shows increased butanol tolerance, it does not contain a homobutanol pathway. Due to the fact that this strain relies on glycolysis for a reducing power supply, further improvements in butanol production may be hindered by a redox imbalance. Other studies have looked at improving butanol tolerance in *E. coli* [15], *Pseudomonas putida* [21], and *Bacillus subtilis* [13] with some success, but these improved strains do not contain the genes required to produce butanol. Nevertheless, it has been reported that cloning *C. acetobutylicum* genes into wild-type *E. coli* yielded a maximum of 4.2 mM (325 mg $l^{-1}$) butanol (*adheI*) and 16.2 mM (1,199 mg $l^{-1}$) butanol (*adheII*) [9]. Again, these strains would require oxidation of two glucose molecules (by glycolysis) to supply the reducing power needed for the production of one butanol. Elimination of *E. coli* competing pathways has been successfully attempted in order to increase the availability of NADH for increased butanol production in the engineered *E. coli* strain [1, 23]. However, the problem of insufficient reducing power supply remains because the engineered *E. coli* host still can only generate 2 NADH per glucose from glycolysis, and alternative products like formate are still being produced. In our study, we not only eliminated the competing pathways but also increased the NADH output by anaerobically expressing the pyruvate dehydrogenase complex, which can generate additional NADH. This improvement enabled the *E. coli* EG03 host to establish a homobutanol pathway once the clostridial butanol pathway genes were cloned in. Although the butanol titer (1,254 mg $l^{-1}$) achieved by *E. coli* EG03 (pEAG13) was not significantly higher than those produced by wild-type *E. coli* harboring a non-homobutanol pathway (1,199 mg $l^{-1}$) [9], the homobutanol pathway of our engineered strain [one glucose (molecular weight of 180) converted to one butanol (molecular weight of 74)] could achieve a theoretical butanol yield of 41 % on a molecular weight basis, which is two-times higher than strains without a homobutanol pathway [two glucose needed (to generate 4 NADH) to produce one butanol]. From a practical-point-view, improvement of the product yield should decrease production costs because substrate costs could account for approximately 30 % of butanol production costs [20]. To this end, however, further improvement of *E. coli* EG03 (pEAG13) should be achieved in at least five

approaches (1) decreasing ethanol production by deleting the *mhpF* and *adhP* genes; (2) minimizing ethanol production by using the butyryl-CoA-specific butanol dehydrogenase (BdhAB) genes rather than the *adheII* gene; (3) enhancing expression of the butanol pathway genes using *E. coli* promoters and ribosomal binding sites; (4) integrating the butanol pathway genes into the *E. coli* chromosome; (5) increasing butanol tolerance of the engineered host through adaptive evolution since the parent strain SZ470 has a limited butanol tolerance (cell growth decreased by 45–50 % with 1 g $l^{-1}$ of butanol) [28].

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