

# One-pot Production of Butyl Butyrate From Glucose by Constructing a “Diamond-Shaped” E. Coli Consortium

## Jean Paul Sinumvayo

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. University of Chinese Academy of Sciences, Beijing 100049,

## Chunhua Zhao

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. University of Chinese Academy of Sciences, Beijing 100049,

## Guoxia Liu

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

## Yanping Zhang

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

## Yin Li (✉ [yli@im.ac.cn](mailto:yli@im.ac.cn))

Institute of Microbiology Chinese Academy of Sciences

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## Research

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# Abstract

Esters are widely used in plastic, texture, fiber, and petroleum industries. Usually, esters are produced from chemical synthesis or enzymatic processes from the corresponding alcohols and acids. Recently, fermentation production of esters was developed with supplementation of precursors (either alcohol or acid, or both at once). Here using butyl butyrate as an example, we demonstrated that we can use a microbial consortium developed from two engineered butyrate- and butanol-producing *E. coli* strains for the production of ester, with the assistance of exogenously added lipase. The synthesizing pathways for both precursors and the lipase-based esterification reaction created a “diamond-shaped” consortium. The concentration of the precursors for ester biosynthesis could be altered by adjusting the ratio of the inoculum of each *E. coli* strain in the consortium. Upon appropriate optimization, the consortium produced 7.2 g/L butyl butyrate without the exogenous addition of butanol or butyrate, which is the highest titer of butyl butyrate produced by *E. coli* reported to date. This study thus provides a new way for the biotechnological production of esters.

## Introduction

Fatty acid esters are one of the large group of value-added chemicals derived from short-chains alcohols and carboxylic acids. They are usually presented in natural sources such as flowers, fermented beverages, and particularly in fruits (Chung et al. 2015; Jenkins et al. 2013). Notably, butyl butyrate (BB) is an ester formed by esterification of butyrate and butanol, and it is known as a flavor and fragrance compound that is widely used in foods, beverages, perfumes, and cosmetic industries (Santos et al. 2007). BB is also an important solvent widely used in the production of plastic, fiber, and processing of petroleum products (Horton and Bennett, 2006; Matte et al. 2016).

Over the years, BB, like other most esters ( $R_1COOR_2$ ), was produced by esterification of butyrate and butanol which is usually catalyzed by inorganic catalysts at relatively high temperature (Ju et al. 2011; Kang et al. 2011). The enzymatic process has also been applied for BB production (Berg et al. 2013; Matte et al. 2016). It should be noted that both current catalytic and enzymatic production of BB require external supplementation of the precursors (butanol and butyrate). Some *Clostridium* species are able to produce butyrate, some can further convert the butyrate produced into butanol. This inspired scientists to use clostridia for BB production. However, during acetone-butanol-ethanol fermentation, most of the butyrate produced during acidogenesis is converted into butanol, leaving little butyrate available for the esterification reaction. Therefore, butyrate, or butanol, or both, need to be added to maintain sufficient levels of precursors (Xin et al. 2019). For example, 7.9 g/L butyrate needed to be supplemented to a fed-batch fermentation of xylose by *Clostridium* sp. strain BOH3 to produce 22.4 g/L BB (Xin et al. 2016), while 10 g/L butanol needed to be added to the fermentation of *Clostridium tyrobutyricum* to achieve a 34.7 g/L BB production (Zhang et al. 2017).

Recently, *Escherichia coli* was engineered to produce BB directly from glucose. An enzyme alcohol acyltransferase (AAT) which is capable of catalyzing the reaction of butanol and butyryl-CoA was

introduced into a butanol-producing *E. coli* strain to produce BB. However, the titer of BB achieved, 47.6 mg/L, is fairly low (Layton and Trinh, 2016). Since BB is produced by esterification of butyrate and butanol, we came up with an idea of developing a microbial consortium for BB production. In this consortium, one strain may produce butyrate, and the other may produce butanol. If a relatively equal amount of butyrate and butanol can be produced at a similar rate, BB can be produced at the presence of lipase, thus achieving one-pot production of BB directly from glucose.

Previously we have developed a chromosomally engineered *E. coli* strain EB243 capable of efficiently producing butanol from glucose (Dong et al., 2017). We intended to construct another butyrate-producing *E. coli* strain by redirecting the carbon flow at the node of butyl-CoA, thus shifting the butanol production to butyrate production. When both strains were co-cultured and supplied with lipase, an *E. coli* consortium capable of producing BB directly from glucose can be constructed (Fig. 1). In this consortium, the two engineered *E. coli* strains share the same metabolism upstream of the butyryl-CoA. The metabolism of the *E. coli* consortium diverges at butyryl-CoA, and converges at BB, thus forming a “diamond-shaped” consortium (Fig. 1). We demonstrate the scientific feasibility of using a microbial consortium for the production of esters with the assistance of exogenously added lipase in a two-liquid-phase fermentation system, providing a new approach for the biotechnological production of esters.

## Materials And Methods

### Strains, plasmids, and primers

*E. coli* EB243 (Dong et al., 2017) was used as the starting strain for further construction of derivative strains. All strains and plasmids used are listed in Table 1. All primers (Table S1) were synthesized by Invitrogen (Beijing, China) and purified via polyacrylamide gel electrophoresis. Candidate genes encoding enzymes for butyrate biosynthesis were successfully amplified by PCR from the *genomic DNA* of *E. coli* for acyl-CoA thioesterase (*yciA*, *tesB*), and *Clostridium acetobutylicum* for phosphate butyryltransferase (*ptb*), butyrate kinase (*buk* and *buk2*), respectively. Acyl-CoA thioesterase gene *yciA* from *Haemophilus influenza* (Menon et al. 2015) was synthesized by GenScript and named as *yciAh* (GenScript, Nanjing, China). Subsequently, each gene was cloned into pAC2 plasmid under miniPtac promoter (Zhao et al. 2019) and independently expressed in strain EB243 $\Delta$ adhE2, resulting in plasmids and strains summarized in Table 1.

### Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
<i>E. coli</i> EB243	Derived from BW25113; Containing butanol synthetic pathway	(Dong et al. 2017)
<i>E. coli</i> EB243Δ <i>adhE2</i>	EB243 derivative, with <i>adhE2</i> deleted	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2	EB243 derivative, harboring plasmid pAC2	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2- <i>ptb-buk</i>	EB243 derivative, harboring plasmid pAC2- <i>ptb-buk</i>	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2- <i>ptb-buk2</i>	EB243 derivative, harboring plasmid pAC2- <i>ptb-buk2</i>	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2- <i>yciAh</i>	EB243 derivative, harboring plasmid pAC2- <i>yciAh</i>	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2- <i>tesB</i>	EB243 derivative, harboring plasmid pAC2- <i>tesB</i>	This study
<i>E. coli</i> /EB243Δ <i>adhE2</i> -pAC2- <i>yciA</i>	EB243 derivative, harboring plasmid pAC2- <i>yciA</i>	This study
Plasmids		
pAC2	pACYC184 derivative , miniPtac, <i>cat</i> , kan <sup>R</sup>	(Zhao et al. 2019)
pAC2- <i>ptb-buk</i>	pAC2 derivative, with genes <i>ptb-buk</i>	This study
pAC2- <i>ptb-buk2</i>	pAC2 derivative, with genes <i>ptb-buk2</i>	This study
pAC2- <i>yciAh</i>	pAC2 derivative, with genes <i>yciAh</i>	This study
pAC2- <i>yciA</i>	pAC2 derivative, with genes <i>yciA</i>	This study
pAC2- <i>tesB</i>	pAC2 derivative, with genes <i>tesB</i>	This study
pTargetF	<i>aadA</i> , guide RNA transcription	(Jiang et al. 2015)
pCas	kan <sup>R</sup> , <i>gam-bet-exo</i> , <i>cas9</i>	(Jiang et al. 2015)
pTargetF- <i>adhE2</i>	Derived from pTargetF, <i>adhE2</i> knockout vector	This study

*cat*: chloramphenicol resistance gene; *aadA*: spectinomycin resistance gene; *kan<sup>R</sup>*: kanamycin resistance gene; *gam-bet-exo*: Red recombinase genes; *cas9*: Cas9 protein coding gene.

## Cell culturing and fermentation conditions

For genetic modification, *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with kanamycin (50 µg/mL) when necessary. Strains were maintained frozen in 15% glycerol at -80°C. Prior to culturing, fresh colonies were picked from LB plates and inoculated directly into LB medium and overnight incubated at 37°C at 200 rpm. Initial optical density for inoculation was set at 0.2 and 0.4 for tube and bioreactor fermentation, respectively. Based on the genotypic modifications made to our strains, oxygen-dependent conditions were applied for tube or bioreactor fermentation.

Tube fermentation was performed in a sealed 10- or 50-ml polypropylene conical tube (BD Biosciences, San Jose, CA) containing various volumes of the medium, which was slightly modified from the previous studies M9Y medium (Dong et al., 2017) (M9 medium + 2 g/L yeast extract + 20 g/L glucose). Cell growth was maintained at a constant shaking speed at 200 rpm at 37°C for 48 h or longer when necessary. Samples were harvested every 24 h, and 0.5 ml of fermentation medium was centrifuged at 12500 rpm. The supernatant was filtrated using organic membrane, transferred into 2 ml HPLC vials, and subsequently subjected to the HPLC for sugar and metabolites analysis.

The bioreactor fermentations were conducted in a 1 L Infors HT bioreactors containing 0.9 L working fermentation medium (M9 medium + 5 g/L yeast extract + 60 g/L glucose) with air blowing. The agitation speed was set to 200 rpm while the pH was maintained at 6.8 by the automatic addition of 5 M NaOH. During the process of fermentation, 1 ml of fermentation culture was taken for analysis every 24 h, where 0.5 ml was used for cell growth monitoring, while the other 0.5 ml was centrifuged at 12500 rpm. The supernatant was filtrated using organic membrane, then transferred into 2 ml HPLC vials, and subsequently subjected to the HPLC for concentrations analysis of butyrate, glucose, and/or butanol where necessary.

### **Production of BB from glucose**

For BB production experiments, 0.75 ml overnight cultures, which contains 0.15 ml butyrate-producing strain EB243 $\Delta$ adhE2::yciAh and 0.6 ml butanol-producing strain EB243 (in a ratio of 1:4), were inoculated in 15 ml M9Y medium in a sealed 50-mL polypropylene conical tube. 5 g/L of LCS (lipase from *Candida* sp. recombinant, expressed in *Aspergillus niger*, in aqueous solution, also named as Novozymes Lipozyme® CALB) (Sigma-Aldrich) was added to the medium after 12 h to convert the butanol and butyrate into BB. 15 ml hexadecane (Sigma-Aldrich) was added to each 50-mL polypropylene conical tube to extract the BB produced. The cultures were incubated in a rotary shaking incubator at 200 rpm, 37°C, for 72 h. Every 24 h, 1 ml of culture was collected for metabolites and sugar analysis, and simultaneously 1 ml hexadecane layer was taken to detect the concentration of BB produced.

### **Genetic manipulation and strain development**

The simultaneous knockout of *adhE2* and the integration of *yciAh* assembled with strong RBS and miniPtac promoter were carried out in the chromosome of strain EB243 by CRISPR/Cas9 method (Jiang et al. 2015). To undertake this, a pTargetF-derivative plasmid harboring a designed N20 DNA sequence

from the genomic target (gene) and the corresponding homologous fragment along with the pCas plasmid, which expresses Cas9 protein and Red recombinase were cotransformed in fresh *E. coli* competent cells. After getting transformants, the right mutants were PCR-based screened and confirmed by DNA sequencing; hence, they were used for the two plasmids curing. Furthermore, inducing pCas with IPTG results in cells free-pTargetF while pCas can be cured by cultivating mutant cells at an elevated temperature since pCas is temperature-sensitive. In our case, for *yciAh* integration, the primers pTargetF-adhE2N20-1/pTargetF-2 were used to amplify pTargetF-adhE2 containing the designed N20 sequence, and the primers adhE2-up-F/adhE2-up-R and adhE2-down-F/adhE2-down-R were used to amplify the homologous arms. On the other hand, *yciAh*-F with adhE2-up-R half homologous sequence and *yciAh*-R with adhE2-down-F half homologous sequence were used to amplify the *yciAh* gene we wanted to integrate chromosomally. Then, the three fragments were fused to form a homologous fragment. Subsequently, pTargetF-adhE2, and the homologous fragment were introduced into strain EB243 harboring pCas. The resulted mutant strain was verified by colony PCR using the primers adhE2-up-F/adhE2-down-R. In the end, we got a plasmid-free strain by applying the curing strain strategy as described above. The integrated sequences were amplified from the constructed pAC2-based plasmids (Table 1), constructed using the Gibson assembly kit (New England BioLabs, Beijing, China).

## Analytical methods

The optical density at 600 nm for cell growth ( $OD_{600}$ ) was tested using a UV-visible light spectrophotometer (UV-2802PC; Unico, Shanghai, China). Determination of the concentrations of butyrate, butanol, and glucose in the fermentation samples was performed by Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA), equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Inc., Richmond, CA) with 5 mM  $H_2SO_4$  as the mobile phase (10  $\mu$ L injection, 0.5 mL/min, 55°C). For BB detection samples were taken from the solvent phase during fermentation and were filtrated and all together immediately analyzed on a GCMS-QP2010 Ultra (Shimadzu, Japan) system equipped with a DB-5ms column (30 m length, 0.25 mm inside diameter, 0.25  $\mu$ m thickness, Agilent, USA). The flow rate of the helium carrier gas was 1 ml/min. The interface and ion source temperatures were set to 250°C and 200°C, respectively. The electron impact voltage was set to 70 eV. The  $m/z$  range was 35-500. The column temperature was initially set to 100°C, after which it was increased to 250°C at a rate of 20°C/min, where it was held for 5 min.

## Statistical analysis

The statistical analysis of data and plots was performed using Student's *t*-test in Origin software. *P* values of <0.05 were considered to indicate statistical significance.

## Results

# Construction of butyrate-producing strains

To construct a butyrate-producing strain, we started from the chromosomally engineered *E. coli* strain EB243 which is capable of efficiently producing butanol from glucose. Since the production of butyrate and butanol diverges at the node of butyryl-CoA, a simple strategy would be blocking the butanol synthesis of strain EB243 while simultaneously introducing a suitable enzyme capable of converting butyryl-CoA into butyrate. Thioesterase, butyrate kinase, and phosphate butyryltransferase are all capable of catalyzing this reaction. Therefore, three acyl-CoA thioesterase genes (*yciA* and *tesB* from *E. coli*, *yciAh* from *H. influenzae*), one phosphate butyryltransferase gene (*ptb* from *C. acetobutylicum*), and two butyrate kinases genes (*buk* and *buk2* from *C. acetobutylicum*) were selected for testing.

The butyrate-producing ability of the starting strain *E. coli* EB243 (Dong et al. 2017) was investigated. It only generates 0.25 g/L butyrate after 72 h fermentation, suggesting a very weak butyrate-producing ability. This is due to the function of aldehyde/alcohol dehydrogenase AdhE2 for butanol production. Therefore, *adhE2* needs to be deleted and genes responsible for butyrate formation need to be introduced. Based on strain EB243, *adhE2* was first deleted to form strain EB243 $\Delta$ *adhE2*. Subsequently, we selected and expressed different genes from various sources to increase the titer and yield of butyrate. The plasmids harboring genes of interest were transformed in *E. coli* DH5 $\alpha$ , checked by colony PCR, and verified by sequencing before transformation into strain EB243 $\Delta$ *adhE2*.

### Production of butyrate in tube and bioreactor fermentation

To test if candidate genes function for butyrate production, three single genes (*yciA*, *tesB*, and *yciAh*) encoding thioesterase and two genes pairs (*ptb-buk*, and *ptb-buk2*) encoding phosphate butyryltransferase and butyrate kinase, respectively, were cloned into pAC2 plasmid then expressed in strain EB243 $\Delta$ *adhE2*. The resulted strains were individually inoculated in 5 mL M9Y medium in a 10 mL polypropylene centrifuge tube with appropriate antibiotics. The strain containing gene *yciAh* from *H. influenzae* manifested the highest butyrate production of 1.06 g/L in 72 h with a yield of 0.29 g/g glucose, while the production of butyrate using strains containing *yciA* or *tesB* from *E. coli*, or *ptb-buk*, *ptb-buk2* from *C. acetobutylicum* were all below 1 g/L (Fig. 2a).

The supply/consumption of NADH is balanced in pathways of strain EB243 which is capable of producing butanol. However, once strain EB243 is engineered from butanol production to butyrate production, the NADH will be in excess conditions. This is because the NADH which is the substrate of aldehyde/alcohol dehydrogenase (encoded by *adhE2*) in strain EB243, can not be recycled in *adhE2*-deleted strain EB243 $\Delta$ *adhE2* and the pathway for butyrate production does not need NADH. To recycle the NADH to continue the fermentation, oxygen needs to be supplied for butyrate production. In fact, strain EB243 $\Delta$ *adhE2* does not grow, nor produce butyrate, under anaerobic conditions (data not shown).

Subsequently, the production of butyrate by the six constructed strains was quantified and compared in a 50 ml polypropylene conical tube containing 5 ml M9Y medium. All strains produced a higher titer of butyrate (Fig. 2b) as compared to the titer observed when the fermentation was performed in a 10 mL polypropylene centrifuge tube (Fig. 2a). Strain EB243 $\Delta$ *adhE2*-pAC2-*yciAh* yielded the highest butyrate production among all recombinant strains, suggesting that improving aeration did benefit butyrate

production. By changing culture volumes, we performed additional tube fermentation experiments to study the effect on butyrate production. It was found that strain EB243 $\Delta adhE2$ -pAC2-yciAh exhibited a maximal titer of 3.5 g/L with a yield of 0.34 g/g glucose when 30 ml M9Y medium is loaded in 50 ml tube (Fig. S1c). This suggests moderate aeration is needed for butyrate production.

### **Batch fermentation for butyrate production**

The *yciAh* gene, which showed to be the best candidate for butyrate production at tube scale fermentation, was integrated chromosomally to obtain an antibiotics-free strain. The resulted strain EB243 $\Delta adhE2::yciAh$  was further subjected to bioreactor fermentation to evaluate the production ability of butyrate. As the butyrate production is strongly related to oxygen availability, the fermentation system was aerated at a rate of 0.3, 0.5, 0.75, and 1 vvm (volume of gas per volume of liquid per minute) (Fig. S2), which resulted in the final OD<sub>600</sub> of 16.2, 17.2, 13.2, and 13.9, respectively (Fig. S2a). As proof of concept, the airflow rate optimized at 0.5 vvm allowed strain EB243 $\Delta adhE2::yciAh$  to produce 12.4 g/L butyrate. Simultaneously, a promising butyrate yield of 0.46 g/g of glucose (93.9 % of the theoretical yield) and a productivity of 0.17 g/L/h were achieved after 72 h which was the highest yield for *E. coli* compared to the recently published work (Wang et al. 2019). A higher airflow (0.75 or 1 vvm) did not favor butyrate production, which confirmed the above results of tube fermentation.

### **Co-production of butanol and butyrate by the consortium**

Using the butyrate-producing strain EB243 $\Delta adhE2::yciAh$  and the butanol-producing strain EB243 (Dong et al. 2017), a microbial consortium was built to produce butanol and butyrate *simultaneously* required for BB biosynthesis. However, the consortium constructed could not produce butyrate under anaerobic conditions, while under aerobic conditions it could produce butyrate but the butanol production would be impaired. Considering the demand of moderate aeration for butyrate biosynthesis as described above, and the relative anaerobic demand for butanol fermentation (Dong et al. 2017), the mismatched oxygen demand would be a challenge for the synchronous production of butyrate and butanol. To address this challenge, we tried to alter the ratio of the butyrate- and butanol-producing strains and investigate whether we can find a suitable ratio that may enable the consortium to produce both butyrate and butanol under moderate aerobic conditions. The ratio of 1:4 (butyrate strain: butanol strain) was shown to be the best for the simultaneous production of butyrate and butanol in tube fermentation. Under such a strategy, the titer of butyrate and butanol reached 2.5 g/L and 2.4 g/L, respectively, as depicted in Fig. 3a and Fig. S3f. It is also interesting to note that butanol production under aerobic conditions is increasing along with increasing the ratio of butanol-producing strain in the consortium, suggesting altering the ratio of the consortium is an effective approach.

### **In situ production of BB using microbial consortium in the presence of lipase**

#### **Batch fermentation for production of BB**

5.0 g/L of Lipase is used for the esterification of butyrate and butanol.

The fermentation was carried out in bioreactors with 0.5 L of M9 modified medium and 0.5 L hexadecane as an extractant so as to in situ remove BB from the aqueous phase to avoid potential inhibition. pH was controlled at 6.4, and 0.5 vvm of air was bubbled through the bioreactor for aerobic growth with an agitation speed of 200 rpm, since mixing the butyrate and butanol thoroughly is essential for the lipase-catalyzed esterification reaction (indicated from Fig. S4). Under these conditions, 1.1 g/L BB was produced at the end of the fermentation (Fig. 4c). At this point, while 7.1 g/L butyrate were still present in the fermentation broth, the residual butanol concentration was only 1 g/L (Fig. 4d). We therefore wondered if the shortage of butanol could be the reason related to the low BB titer. Besides, as pH may affect the dissociation status of butyrate, the effect of pH should be further investigated.

### **Optimization of pH control for improved BB production**

It is reported that butyrate is present in undissociated form when the pH of the fermentation medium is low which may favor the esterification of butyrate with butanol to produce BB (Harroff et al. 2019; Zhang et al. 2017). But low pH may severely impair bacterial cell growth and lead to poor fermentation performance (Maddox et al. 2000).

Therefore, we investigated the effect of pH on the production of BB by the consortium in the bioreactor.

At pH 4.5, and 5, the consortium could not grow (data not shown), while at pH 5.5, the microbial consortium could grow moderately, however, there were neither butyrate nor butanol produced (Fig. 4d) which is closely related to the poor growth of strains, leading to the slow utilization of glucose and high residual concentration (Fig. 4b). This means that pH lower than 5.5 is not suitable for the simultaneous biosynthesis of butyrate and butanol in *E. coli* (Fig. 4). To tackle this challenge, a parallel experiment was performed at pH 5.9. The consortium was able to grow well (Fig. 4a), and almost all the glucose were consumed (Fig. 4b). 2.2 g/L butyrate along with 1.0 g/L butanol remained in the fermentation medium at the end of fermentation. Overall, the consortium produced 5.1 g/L BB.

All parameters were assessed at pH 5.5, 5.9, and 6.4 Butyrate, OD600, butanol, butyl butyrate, and glucose were detected every 24 hours. The experiments were performed in three biological replicates.

### **Increasing the ratio of butanol-producing strain favored BB production**

From the above studies, butanol concentrations in the fermentation broth were always lower than those of butyrate. Therefore, we hypothesized that BB biosynthesis might be limited by the insufficient supply of butanol. In this case, more butanol-producing cells were inoculated with a ratio of 1:8 (butyrate strain: butanol strain). Comparing with the control consortium inoculated at a ratio of 1:4 (butyrate strain: butanol strain), it is evident that the consortium inoculated at a ratio of 1:8 grew significantly better (Fig. 5a). High concentration of butanol was accumulated (Fig. 5b), which is associated with increased butanol cells in the fermentation when the consortium was inoculated at the ratio of 1:8. The highest titer of BB, 6.1 g/L, was obtained after 72 h, but the yield on glucose (0.09 g/g) is very low. The very low yield

of BB on glucose could be related to the supply of oxygen, as excess glucose might be respired under aerobic conditions. We therefore further investigated the effect of aeration on BB production.

### **Regulating the aeration for improving BB biosynthesis**

When the consortium is grown at aerobic condition, 0.5 vvm, butyrate was produced up to 5.9 g/L in 24 h of fermentation while butanol only reached 1.9 g/L under the same condition. This indicates that butanol producing strain do not work efficiently under aerobic condition, as expected. However, as described before anaerobic conditions do not favor butyrate production either. Since our previous research on butanol production also applies microaerobic conditions in the beginning of fermentation to facilitate cell growth, we wonder whether we can develop a two-stage aeration strategy for BB production. In this two-stage strategy, relatively high aeration is given in the first stage to satisfy the growth of both butyrate- and butanol-producing strains, also to facilitate butyrate production. Subsequently, the aeration might be decreased at a certain time point to favor butanol production, while still able to sustain butyrate production. We expect such a two-stage aeration strategy might achieve a better match for the different oxygen demand of butyrate- and butanol-producing strains, thus achieving a higher BB production.

Here we grew the cells at aeration of 0.5 vvm in the first stage of the fermentation for 24 h (strategy 1) or 36 h (strategy 2) and then shifted the aeration to 0.1 vvm in the second stage of the fermentation up to 72 h. As shown in Fig. 6d, the consortium grown under strategy 2 produced 7.2 g/L BB with a yield of 0.12 g/g of glucose, compared to 6.35 g/L BB with a yield of 0.11 g/g of glucose when the consortium grew under strategy 1. Interestingly, the consortium grown under strategy 2 was still able to maintain the momentum to produce butanol and butyrate, compared with the consortium without two-stage aeration control. The results demonstrated that the approach not only increased the BB titer but also slightly increased the yield. However, the yield obtained is much lower than the theoretical yield of BB on glucose (0.4 g/g), most likely related to aeration which needs further study.

**C:** aerated with 0.5 vvm for the whole fermentation process

**1:** aerated with 0.5 vvm in the first 24h, then shifted to 0.1vvm

**2:** aerated with 0.5 vvm in the first 36h, then shifted to 0.1vvm

The data represent the means  $\pm$  SD from three biological replicates.

## **Discussion**

Considering various or versatile applications of BB and troublesome in its production using microorganisms, many efforts have devoted to develop different strategies for improving BB production. *Naturally, clostridial strains are thought to be good candidates for BB production as some species produce butyrate and some produce butanol. Usually, the butanol produced by clostridial strains is converted from butyrate. This makes it difficult to maintain a reasonable ratio of butyrate and butanol*

during the fermentation of clostridial strains. Thus, butyrate or butanol needs to be supplemented (Xin et al. 2019).

In this study, we developed a one-pot process for direct production of BB from glucose by engineered *E. coli* strains, without the addition of exogenous butyrate and/or butanol. This was achieved by segregating the synthetic pathway for butyrate and butanol in two *E. coli* strains that otherwise have the same genetic background. A combination of the butyrate- and butanol-producing *E. coli* strains formed a “diamond-shaped” consortium capable of producing BB in the presence of lipase and solvent. *E. coli* is not a natural strain for butyrate production, nor for butanol. Based on the chromosomally engineered *E. coli* strain EB243 capable of efficiently producing butanol obtained in our previous work (Dong et al., 2017), we were able to engineer strain EB243 into a chromosomally engineered strain EB243 $\Delta$ adhE2::ycaAh capable of producing 12.4 g/L butyrate with a yield of 0.46 g/g on glucose in batch bioreactor fermentation. Using the efficient butyrate-producing strain constructed in this work, and the efficient butanol-producing strain developed in our previous work, we were able to construct a consortium of strains derived from the same *E. coli*. Since these two *E. coli* strains in the consortium are nearly identical with only a few genes difference, the ratio of the two strains can be easily adjusted to obtain different production of butyrate and butanol. Upon optimization of the ratio of these two strains, optimization of pH, and using a two-stage aeration strategy, 7.2 g/L BB was produced directly from glucose by this consortium during batch fermentation. Nevertheless, the yield on glucose (0.12 g/g) is rather low, implying there is huge room for further improvement.

Recently, *E. coli* was also engineered for *in vivo* biosynthesis of esters. However, all strains developed to date showed a very low titer of the target esters in the fermentation broth. In one study, alcohol acyltransferase (AAT) from *Fragaria ananassa*, a cultivated strawberry, was appropriately expressed in *E. coli*, and subsequently cultured with exogenous BB precursors. However, only 0.28 mg/L BB was produced when 1 g/L butanol and 3 g/L butyryl-CoA were added to the system (Horton and Bennett, 2006). In 2014, various modules related to the production of alcohols, along with an alcohol O-acyltransferase (ATF1) from *S. cerevisiae* known to catalyze the last step of ester biosynthesis, were designed and introduced in *E. coli*. However, of all the esters produced in the mixture, there was no BB observed. Arguing that this strategy could provide butyryl-CoA, the process was fed with 3 g/L of butanol, which resulted in a low titer of 14.9 mg/L BB (Rodriguez et al. 2014). Another study aimed to engineer *E. coli* to produce BB via fermentative biosynthesis (Layton and Trinh 2014). In this study, the enzymatic ester pathway with the AAT submodule from *Fragaria ananassa* were introduced in *E. coli* to generate alcohol and acyl-CoAs molecules. However, among esters produced, there was no BB detected, which may have been due to the lack of butanol in the system. Upon addition of 2 g/L butanol, BB could be produced but with a low titer of 36.8 mg/L. In an additional study, the prospect of carboxylate to ester platforms has been demonstrated. To implement this, an engineered *E. coli* modular chassis cell was tightly assembled with modular heterologous pathways comprising an acid to acyl-CoA synthesis submodule (acyl CoA transferase), an acyl CoA and alcohol condensation submodule (alcohol acyltransferase), and an alcohol production submodule which subsequently co-fermented with glucose to form a combinatorial biosynthesis of fermentative esters. 2 g/L butyrate was supplemented to the

fermentation medium to reinforce the CoAs molecules accumulation. Finally, only 47.6 mg/L BB was produced (Layton and Trinh, 2016).

Production of BB by engineered *E. coli* remains low (in mg/L levels) even under the supplementation of exogenous substrates. The reason could be ascribed to insufficient information on the characteristics of alcohol acyltransferase. Besides, the biotechnological production of BB has a close link with the intrinsic precursors such as butyryl-CoA and butanol. Preferably, both substrates should have been produced in a ratio of 1: 1 for efficient conversion of sugars into BB. However, this is a problem in *E. coli* because butyryl-CoA and butanol are produced in a complex and interlinked metabolic pathway, making their ratio balance a challenge. The way we deal with this challenge is to develop a consortium comprising separate engineered butyrate- and butanol-producing strains. However, achieving stable and reproducible ratios for different strains as well as their performance is still challenging, especially for microbial consortia where mutual beneficial interactions are missing - for example, if one strain grows under aerobic conditions while another prefers anaerobic conditions.

In our study, the anaerobic butanol-producing strain used in this study requires 2NADH in its last steps to produce butanol. This strain was also used to construct a butyrate-producing strain by deleting gene responsible for butanol production then integrating genes responsible for butyrate production. However, this strategy leaves 2NADH unoxidized, which forces the butyrate-derived strain to grow on aerobic growth. Since butyrate- and butanol-producing *E. coli* strains were required to construct a “diamond-shaped” consortium for efficient BB production, optimum aeration to favor both strains to produce balanced precursors, should be taken into account. We noticed that the shortage in the precursor, for example, butanol in this study, was a limiting factor for BB biosynthesis. In this case, we split the fermentation process into two different aeration stages, which enabled the strains to produce precursors as well as maintaining the “diamond-shaped” consortium stability (Fig. 6d). When the aerobic condition was applied in the first 24 h (strategy 1), the excess NADH could be oxidized, resulting in butyrate production. Since the consortium requires butanol and given that butanol strain does not tolerate aeration, turning the consortium to the anaerobic stage would have favored the consortium to produce butanol as long as the fermentation progresses. Nevertheless, the BB (6.35 g/L) produced was not increased significantly compared to the BB (6.1 g/L) produced by the control strategy where the entire process was kept aerobic. When the aerobic stage applied in the first hours to favor butyrate production was increased up to 36 h (strategy 2), the BB produced increased up to 7.2 g/L as well. This shows adjusting the mode of aeration supply may improve the balanced production of butyrate and butanol in the fermentation, hence leading to improved BB biosynthesis.

## Conclusion

In this study, we developed a cognate *E. coli* consortium for direct production of butyl butyrate from glucose in a one-pot process. The cognate *E. coli* consortium comprises a butyrate-producing strain and a butanol-producing strain which share the same pathway upstream of the butyryl-CoA. The nearly identical genotype of these two strains lightens the need for manipulation of nutritional conditions for

this consortium. The cognate consortium could produce 7.2 g/L butyl butyrate from glucose under suitable conditions without the exogenous addition of butanol or butyrate. This is the highest titer of butyl butyrate directly produced from glucose by *E. coli* reported to date, indicating the potential of using *E. coli* strain for biological production of esters.

## Abbreviations

**BB:** Butyl butyrate

**CoA:** Coenzyme A

**S1:** glucose

**S2:** acetyl-CoA

**Sn:** acetoacetyl-CoA

**LCS:** lipase from *Candida* sp. recombinant, expressed in *Aspergillus niger*, in aqueous solution, also named as Novozymes Lipozyme® CALB

**CRISPR:** Clustered Regularly Interspaced Short Palindromic Repeats

**GCMS:** Gas chromatography–mass spectrometry

**HPLC:** High Performance Liquid Chromatography

**NADH:** Nicotinamide Adenine Dinucleotide

**SD:** Standard Deviation

**OD:** Optical Density

**pH:** Potential of Hydrogen

**AAT:** Alcohol Acyltransferase

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

## Availability of data and material

The supporting document showing the findings of this study are available from the corresponding author at any time required.

## Competing interests

The authors declare no conflict of interest.

## Funding

Not applicable.

## Author's contributions

JPS, CZ, YZ and YL conceived and designed the study. JPS, CZ and GL performed the experiments.

JPS analyzed the data. JPS, YZ, and YL wrote the paper. All authors read and approved the final manuscript.

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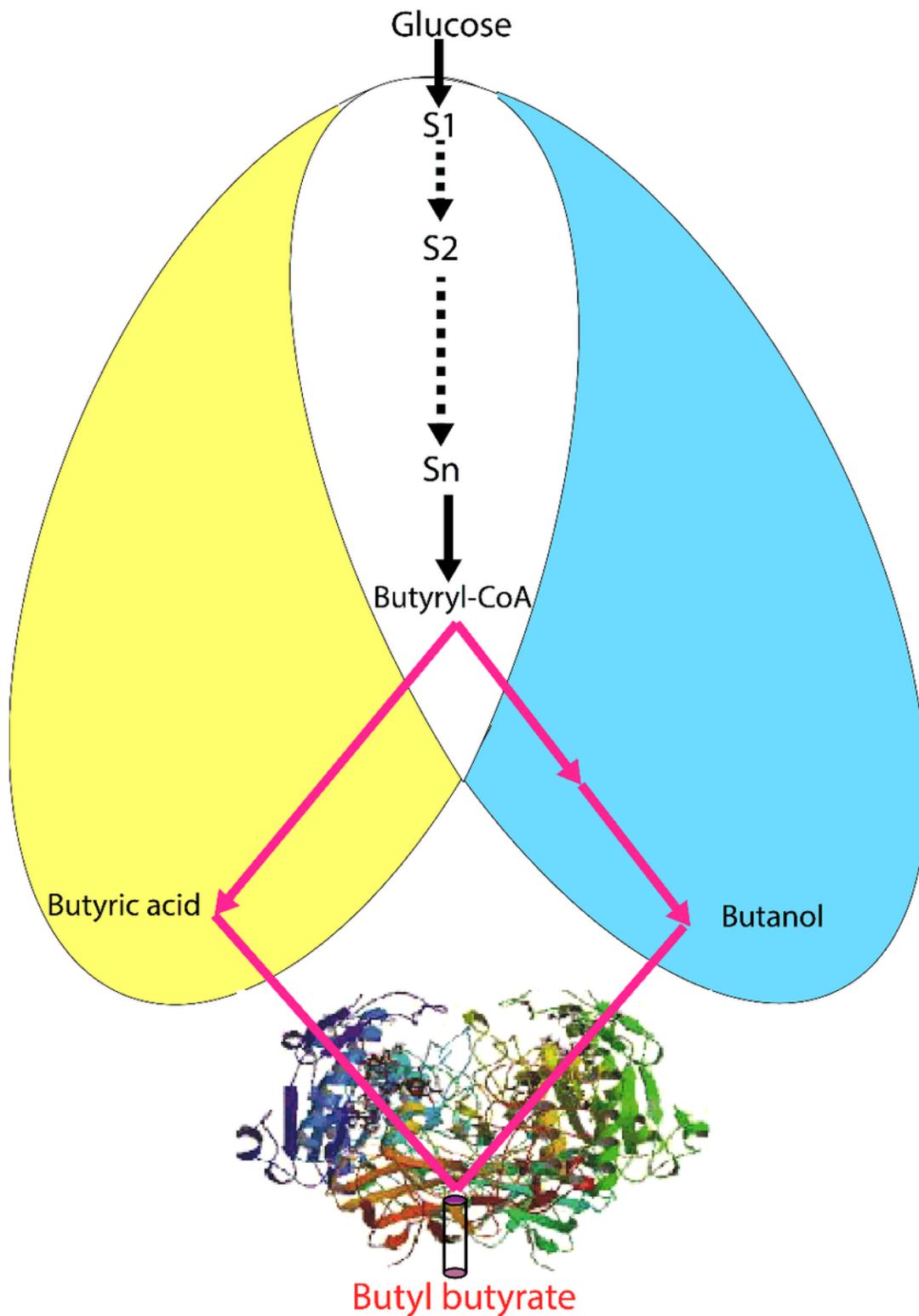
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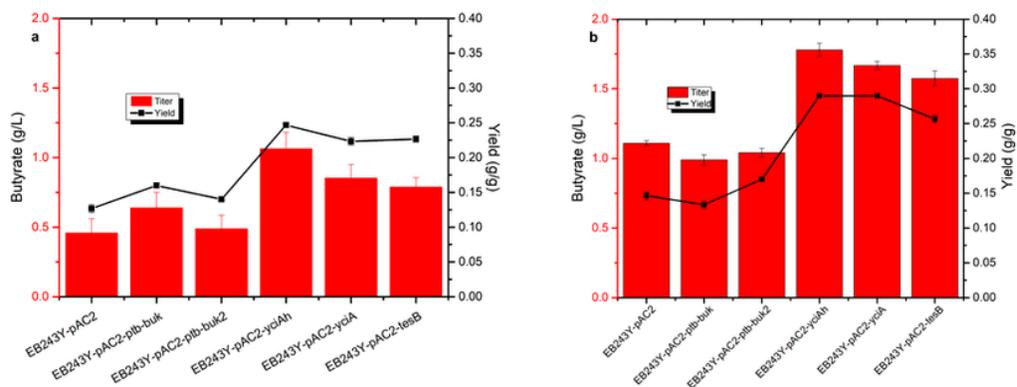
## Figures



**Figure 1**

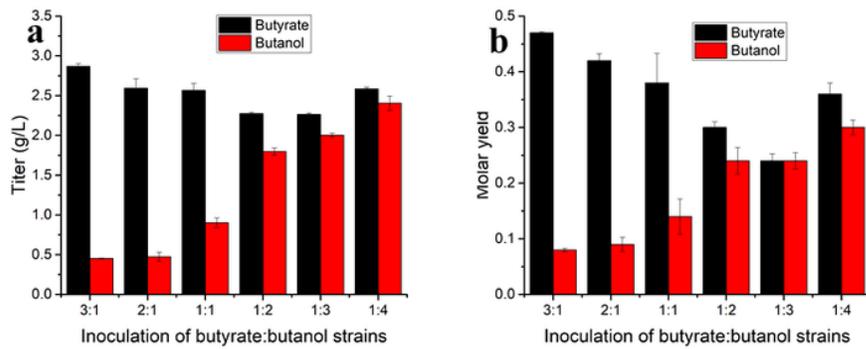
Construction of a “diamond-shaped” consortium to produce butyl butyrate from glucose using two *E. coli* strains. Two engineered *E. coli* strains employed to develop a consortium are denoted by light yellow color for the butyric acid-producing strain and light blue color for the butanol-producing strain. A shared pathway from glucose to butyryl-CoA is shown by a light grey color in the middle of the two strains. A diamond-shaped in red color represents the production routes of precursors (butanol and butyric acid)

and their conversion into butyl butyrate with the assistance of lipase enzyme. S1, S2, and Sn represent glucose, acetyl-CoA, and acetoacetyl-CoA, respectively.



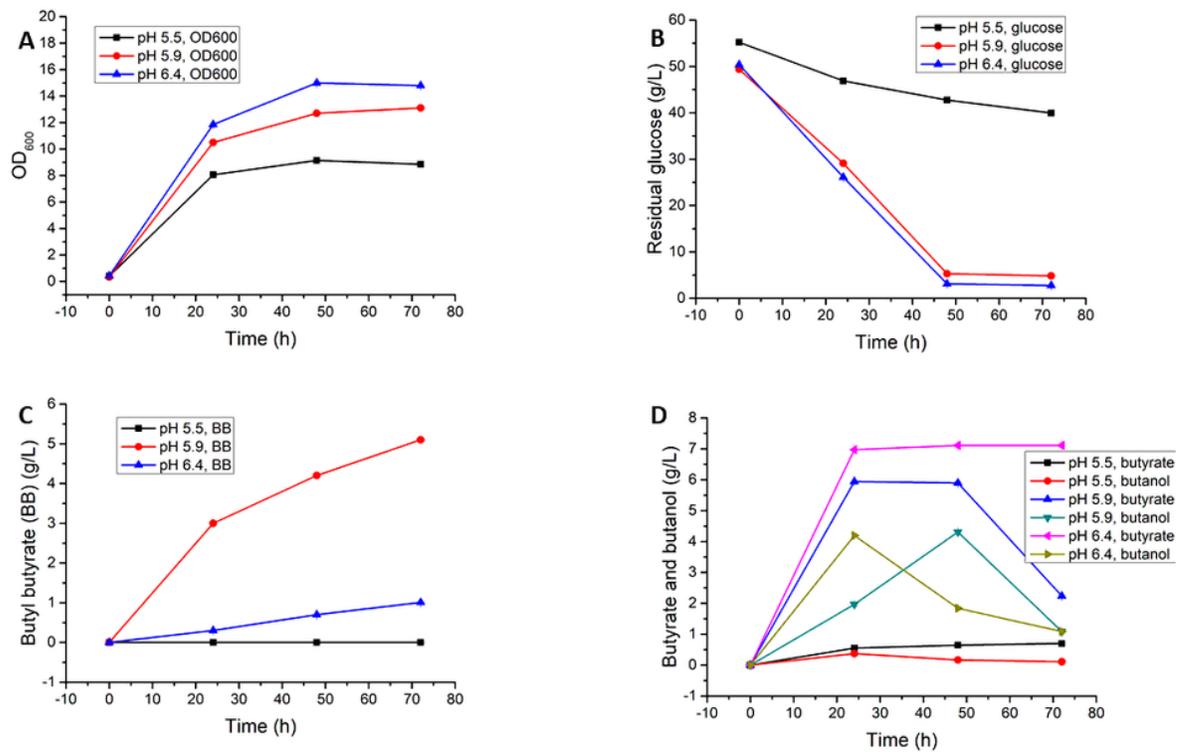
**Figure 2**

Investigation of butyrate production by overexpressing different butyrate-biosynthesis genes. a Strains were cultured in a 10 mL polypropylene centrifuge tube containing 5 ml M9Y medium, while for b strains were cultured in a 50 mL polypropylene centrifuge tube containing 5 ml M9Y medium. “Y” stands for  $\Delta$ adhE2. “yciAh” represents yciA from Haemophilus influenza. The data represent the means  $\pm$  SD from three biological replicates.



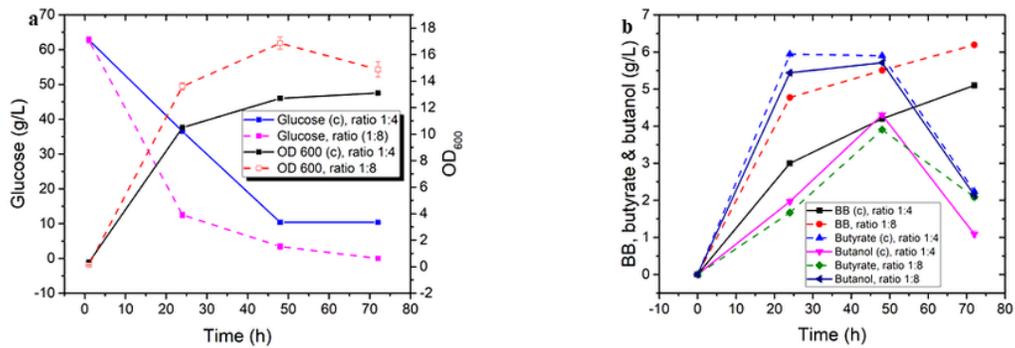
**Figure 3**

Production of butyrate and butanol by varying inoculation ratios of butyrate-to butanol-producing strains. Titrers (a) and yields (b) of butyrate and butanol were obtained at 72 h of tube fermentation. The data represent the means  $\pm$  SD from three biological replicates.



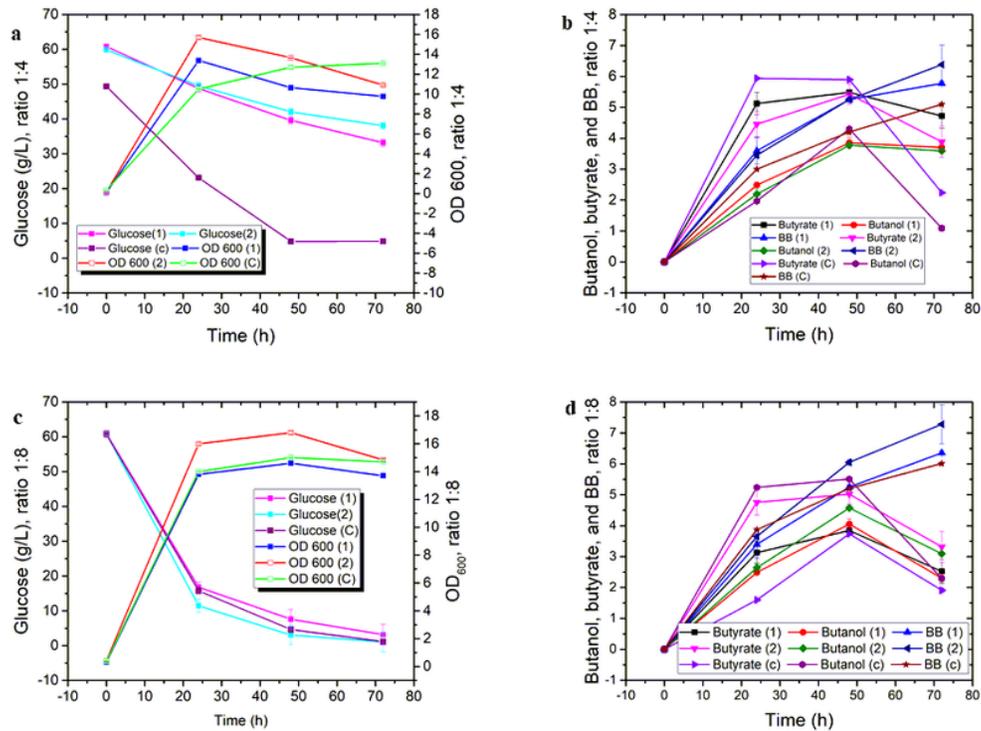
**Figure 4**

Batch bioreactor fermentation profiles of the two strains for butyl butyrate production at different pH. Strains were inoculated at a ratio of 1:4 (butyrate-producing strain (EB243ΔadhE2::yciAh): butanol-producing strain (EB243) in M9Y modified medium supplemented with 50 g/L glucose. The fermentation system was set at a ratio of 1:1 (fermentation broth: hexadecane as extractant). a Optical density (OD 600), b glucose consumption, c butyl butyrate biosynthesized during fermentation, and d butyrate and butanol. All parameters were assessed at pH 5.5, 5.9, and 6.4 Butyrate, OD600, butanol, butyl butyrate, and glucose were detected every 24 hours. The experiments were performed in three biological replicates.



**Figure 5**

Fermentation profiles of the microbial consortium for butyl butyrate production responding to the inoculation ratios (butyrate-producing strain: butanol-producing strain) of 1:4 (control, solid line) and 1:8 (dash line). a Profiles of optical density (OD600) and glucose consumptions. b Profiles of butyrate, butanol, and butyl butyrate. The experiments were performed in three biological replicates.



**Figure 6**

Batch fermentation profiles of the microbial consortium for butyl butyrate production with different aeration speed control strategies. The consortium strains were inoculated with a ratio of 1:4 (butyrate strain: butanol strain), and besides, another consortium strains were inoculated with a ratio of 1:8 (butyrate strain: butanol strain). All consortium strains were cultured at 200 rpm, pH 5.9.

## Supplementary Files

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