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Two-stage process and strain engineering for continuous bioconversion of CO₂ to butanol

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Highlights

- A continuous CO₂-to-butanol process was developed using a two-stage chemostat system.
- *Sporomusa ovata* produced 0.4 g L⁻¹ h⁻¹ acetate in continuous culture.
- Metabolic engineering of *E. coli* improved butanol production 3.8-fold.
- The two-stage system produced butanol at 130 mg L⁻¹ and 4.8 mg L⁻¹ h⁻¹.
- Butanol selectivity was identified as the key bottleneck for economic feasibility.

Keywords

CO₂-to-Butanol biosynthesis, Cascade chemostat, Metabolic engineering, Biofuel

ABSTRACT

The efficient valorization of gaseous C1 feedstocks, such as CO₂, into liquid fuels presents a significant process engineering challenge. Here, we design and demonstrate an integrated chemostat system for the continuous synthesis of butanol from CO₂ and H₂ in a tandem process. Our system architecture sequentially couples two bioreactors housing complementary microorganisms. The first stage utilizes *Sporomusa ovata* to produce acetate from CO₂ and H₂ autotrophically, which then serves as the sole carbon source for metabolically engineered *Escherichia coli* in the second stage. Multi-level metabolic engineering of the biocatalyst resulted in a butanol titer of 422 ± 4 mg L⁻¹ in batch cultures. The two-stage continuous system serves as a proof-of-concept, producing 4.8 mg L⁻¹ h⁻¹ of butanol from CO₂ via acetate as an intermediate. While not yet meeting established economic benchmarks, this study reveals critical optimization targets and establishes a foundational and scalable framework for converting CO₂ to butanol.

1. Introduction

Rising atmospheric CO₂ levels and the increasing demand for sustainable energy solutions pose one of the greatest challenges facing contemporary society. Anthropogenic CO₂ emissions,

primarily from the combustion of fossil fuels, are the principal driver of global climate change, with profound environmental, economic, and societal consequences (Attanayake et al., 2024). Global CO₂ emissions from fossil fuels reached a record high of 37.4 GtCO₂ (gigatons of carbon dioxide) in 2024, representing a 0.8% increase from the previous year, a historical record high (Friedlingstein et al., 2024). In response to this crisis, Carbon Capture and Utilization technology, which captures greenhouse gases from industrial processes and converts them into useful chemicals or fuels, has emerged as a key strategy for a sustainable circular carbon economy (Nagireddi et al., 2023; Sieborg et al., 2024). Acetogenic bacteria represents a distinctive class of obligately anaerobic microorganisms for sustainable carbon fixation and chemical production. These facultative autotrophs use the highly conserved Wood-Ljungdahl pathway—the most energetically efficient CO₂ fixation mechanism—to convert CO₂ into acetate. This biochemical architecture enables acetogens to directly utilize industrial off-gases as feedstocks (Lee et al., 2022; Takors et al., 2018).

Acetate produced by acetogens serves as a promising platform for bio-based chemicals, which can be upgraded into more industrially versatile products (Gong et al., 2022). In this context, butanol, a C₄ alcohol, is a highly attractive target product. Butanol can be blended into diesel and biodiesel fuels without engine modification, and has demonstrated better performance than lower alcohols in enhancing fuel properties, making it a promising next-generation biofuel (Atmanli, 2016). Moreover, a recent techno-economic assessment estimates the selling price of bio-based butanol made from CO₂ to be as low as \$3.4 L⁻¹, depending on process parameters and metabolic efficiency (Adams & Clark, 2024). Although the current production cost of bio-based butanol remains higher than that of conventional fuels, its potential as a high-value biofuel additive justifies

continued development, particularly in the context of sustainable bioprocessing.

Clostridium acetobutylicum represents the benchmark for high-titer acetone-butanol-ethanol (ABE) fermentation, reaching titers of 19.7 g L⁻¹ in batch (Du et al., 2022) and 113.3 g L⁻¹ in fed-batch operation (Xue et al., 2012). However, these processes rely on sugar substrates, as *C. acetobutylicum* does not natively utilize C1 substrates and lacks a CO₂-fixation pathway. Furthermore, acetone and ethanol are inherently co-produced, which limits carbon selectivity toward the target product. Alternatively, autotrophic platforms like *Clostridium carboxidivorans* generally suffer from low selectivity, slow growth, and limited genetic tractability (Phillips et al., 2015). Therefore, it is difficult for a single microorganism to efficiently perform the entire process from C1 gas fixation to the production of complex compounds (Stark et al., 2022).

Producing butanol from CO₂ via acetate is an innovative approach that can simultaneously achieve greenhouse gas reduction and sustainable biofuel production. Acetogens excel at CO₂ gas fixation via the ATP-limited Wood–Ljungdahl pathway, which restricts their ability to produce complex molecules (Fast & Papoutsakis, 2012). Despite recent progress in genetic tools, engineering acetogens remains challenging (Liew et al., 2022; Zhang et al., 2024). By comparison, model organisms like *E. coli* are genetically tractable but cannot utilize C1 gases (Puiggené et al., 2025).

Here, we develop and demonstrate a novel chemostat system that leverages the two functionally distinct bacteria and directly converts CO₂ into butanol (**Fig. 1**). This two-stage process consists of two continuous bioreactors (BR1 and BR2). In BR1, *Sporomusa ovata* is maintained in continuous culture with CO₂ and H₂, converting gaseous CO₂ into acetate. The

acetate-rich effluent from BR1 is then fed directly into BR2, which contains an engineered *E. coli* strain that converts the acetate into butanol through a multi-enzyme synthetic pathway. The *E. coli* strain was engineered through a multifaceted strategy involving the enhancement of acetate assimilation by upregulating native *E. coli* genes (e.g., *acs*, *ackA-pta*), introducing heterologous gene modules for butanol synthesis (e.g., *crt/ccr* and *phaJ/ter*) with varied GC contents (50% or 66%), and inserting redox-balancing genes (e.g., *fdh*, *pntAB*). Bottle-scale cultivation was employed to evaluate these modifications and select the most effective strains for use in continuous culture.

Our work thus aims to (1) establish a stable acetate supply by optimizing the continuous culture of *S. ovata*; (2) construct and improve the productivity of butanol in *E. coli* through multifaceted metabolic engineering, including enhanced acetate assimilation and redox balancing; and (3) implement the integrated process to identify system bottlenecks and optimize continuous production. Furthermore, we invoke a previously conducted techno-economic analysis (TEA) to evaluate the commercial potential and economic competitiveness of this platform in comparison to fossil-derived fuels (Adams & Clark, 2024). By coupling experimental validation with a rigorous economic framework, this work establishes technical feasibility and evaluates the economic competitiveness of a CO₂-to-butanol platform, offering a scalable route toward carbon-neutral fuels.

2. Materials and methods

2.1. Reagents and Materials

Ampicillin, chloramphenicol, kanamycin, and spectinomycin were obtained from RPI (Mt.

Prospect, IL, USA). L-arabinose, acetyl-, acetoacetyl-, 3-hydroxybutyryl-, crotonyl-, butyryl-, and glutaryl-CoA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacto™ yeast extract was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Plasmid DNA purification was conducted using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). *Escherichia coli* DH5α (New England Biolabs, Ipswich, MA, USA) was used as the host for cloning. For cloning, the In-Fusion HD (Takara Bio, Shiga, Japan) and Gibson Assembly Master Mix (New England Biolabs) were utilized. All restriction endonucleases were sourced from New England Biolabs.

2.2. Cultivation of *Sporomusa ovata*

Sporomusa ovata (*S. ovata*; DSM 2662) was obtained from the American Type Culture Collection (ATCC) (Cestellos-Blanco et al., 2021). For pre-cultivation, *S. ovata* was grown heterotrophically in an anaerobic Balch-type tube containing an anaerobic medium and a gas mixture of 80% N₂ and 20% CO₂ at 25 psi and 34°C. The heterotrophic pre-cultured medium composition was as follows (per liter): FeSO₄, 2 mg; Na₂SeO₃, 18 µg; Na₂WO₄·2H₂O, 4 µg; NaCl, 2.25 g; MgSO₄·7H₂O, 500 mg; NH₄Cl, 500 mg; CaCl₂·2H₂O, 250 mg; K₂HPO₄, 348 mg; KH₂PO₄, 227 mg; NaHCO₃, 4 g; yeast extract, 2 g; betaine, 5 g; casitone, 2 g. The medium was supplemented with reducing agents (L-cysteine hydrochloride hydrate, 250 mg L⁻¹; Na₂S·9H₂O, 250 mg L⁻¹), a trace metal solution SL-6 (containing, per liter: ZnSO₄·7H₂O, 0.1 mg; MnCl₂·4H₂O, 0.03 mg; H₃BO₃, 0.3 mg; CoCl₂·6H₂O, 0.2 mg; CuCl₂·2H₂O, 0.01 mg; NiCl₂·6H₂O, 0.02 mg; Na₂MoO₄·2H₂O, 0.02 mg; Na₂WO₄·2H₂O, 0.03 mg) and a vitamin solution (containing, per liter: biotin, 0.02 mg; folic acid, 0.02 mg; pyridoxine hydrochloride, 0.1 mg; thiamine hydrochloride, 0.05 mg; riboflavin, 0.05 mg;

nicotinic acid, 0.05 mg; calcium pantothenate, 0.05 mg; vitamin B₁₂, 0.001 mg; 4-aminobenzoic acid, 0.05 mg L; and (±)-α-lipoic acid, 0.05 mg). After two days of cultivation, the culture was transferred to an anaerobic bottle containing the identical medium composition. Following two days of growth, cells were harvested and washed under strictly anaerobic conditions. The washed cell suspension was subsequently inoculated into a sterile bioreactor for autotrophic cultivation.

2.3. Autotrophic gas fermentation for continuous acetate production

Gas fermentation was performed in a BioFlo® 120 bioreactor (Eppendorf, CT, USA). Continuous gas supply consisted of 80% H₂ and 20% CO₂ at 6 L h⁻¹ and N₂ at 18 L h⁻¹. The off-gas was directed through a condenser and subsequently passed through a sterile 0.22-μm filter before being vented to the atmosphere. The reactor was maintained at atmospheric pressure, with the exhaust line kept open throughout the experiments to prevent pressure accumulation. At the total gas flow rate of 24 L h⁻¹ and a headspace volume of 400 mL, the gas residence time in the headspace is estimated to be 0.02 h, indicating rapid gas turnover while ensuring near-atmospheric pressure through continuous venting. The pH was maintained at 6.8 by automatic titration with 5 M anoxic NaOH, and the temperature was controlled at 34°C. Prior to inoculation, the sterile medium was purged with H₂, CO₂, and N₂ for at least 10 hours to establish anaerobic conditions. The sterile *S. ovata* yeast medium used for autotrophic fermentation was based on the pre-culture medium but was modified to contain adjusted concentrations of reducing agents (125 mg L⁻¹ each), NaHCO₃ (0.8 g L⁻¹), and yeast extract (4 g L⁻¹), and was supplemented with an antifoaming agent (0.3 mL L⁻¹). The full composition is detailed in **Supplementary Information; Table S1**.

To establish a continuous stirred-tank bioreactor, the system was connected to two anaerobic

glass bottles: one containing fresh sterile medium and the other designated for effluent collection. Medium exchange was facilitated via peristaltic pumps. Initially, the bioreactor was operated in batch mode (pH 6.8, 34°C, gas flow rates of 6 L h⁻¹ for H₂/CO₂ and 18 L h⁻¹ for N₂) to allow for sufficient biomass accumulation. After approximately 24 hours, continuous operation was initiated by activating the pumps to simultaneously supply fresh medium to the reactor and transfer culture broth into a second continuous stirred-tank bioreactor. Biomass concentration of *S. ovata* was calculated from OD_{545nm} using the correlation determined in **Note S1**. Acetate concentrations in the reactor broth were quantified by NMR as described in **Note S2**.

2.4. Construction of plasmids and engineered *E. coli* strains

The constructed library of strains used in this study is listed in **Table 2**. All plasmids and primers were synthesized by IDT and listed in **Table S2 and Table S3**. Gene fragments were assembled using Gibson Assembly (New England Biolabs) or In-Fusion HD cloning (Takara Bio) and cloned into plasmid backbones with appropriate antibiotic resistance markers. All constructions were verified by colony PCR and Sanger sequencing prior to transformation.

The butanol biosynthesis pathway was constructed by assembling *phaA* and *phaB* from *Cupriavidus necator* H16. Two gene combinations were used for the conversion of 3-hydroxybutyryl-CoA to butyryl-CoA: one comprising *crt* from *Clostridium acetobutylicum* and *ccr* from *Streptomyces collinus*, and another comprising *phaJ* from *Aeromonas caviae* and *ter* from *Treponema denticola*. The final conversion of butyryl-CoA to butanol was catalyzed by *adhE2* from *C. acetobutylicum* (**Table S4**). These combinations were used to construct strains BW1 through BW4, with codon variants at 50% or 66% GC content (**Table 2**).

Acetate assimilation genes were introduced into strain BW4 by transforming *ackA-pta*, *acs*, or *acsL641P* under the control of an arabinose-inducible promoter, yielding strains BW5, BW6, and BW7. Promoter engineering was performed by replacing the arabinose promoter in pCM271 with the synthetic constitutive promoter J23100, generating strain BW5e. For redox balancing, *fdh* gene from *Candida boidinii*, encoding formate dehydrogenase (*fdh*), was cloned into either the high-copy (pET-*fdh*) or moderate-copy (pCDF-*fdh*) plasmid, generating strains BW5b and BW5c. Additionally, *fdh* was introduced into BW5e to construct BW5g. The *E. coli* native transhydrogenase gene *pntAB* was PCR-amplified from genomic DNA and cloned into BW5b and BW5g, providing strains BW5h and BW5j, respectively.

2.5. Cultivation of *E. coli* strains for butanol production

Cultures of the engineered *E. coli* strains were prepared by overnight growth in LB medium with appropriate antibiotics at 37°C with shaking at 200 rpm. Each culture was then inoculated into a 125-mL serum bottle with *S. ovata* yeast medium containing 8 g L⁻¹ sodium acetate to an OD₆₀₀ of 0.2. Butanol producing genes were induced at an OD₆₀₀ of 0.4-0.5 with the addition of 0.02% (w/v) L-arabinose. The serum bottle was sealed with a butyl rubber stopper and an aluminum crimp to establish microaerobic conditions. The culture was incubated at 30°C with shaking at 150 rpm. Samples were collected every 24 hours during cultivation to monitor acetate consumption and butanol production using HPLC. Biomass concentration of *E. coli* was calculated from OD_{600nm} measurements using the correlation determined in **Note S1 and Fig. S1**. Acetate and butanol concentrations were measured by HPLC as described in **Note S2**.

2.6. Cultivation of engineered *E. coli* in a stirred-tank bioreactor

For cultivation in the bioreactor, an inoculum of the engineered *E. coli* was prepared by first growing a culture from a frozen glycerol stock overnight in LB medium containing the appropriate antibiotics at 37°C, 200 rpm. This primary culture was then sub-cultured into LB medium in a 250-mL flask and grown for 4 hours to an exponential phase OD₆₀₀ of 1.5. The cells were harvested by centrifugation (3,320 x g, 10 min, 4°C) and washed once with *S. ovata* yeast medium to remove any residual LB components. The fermentation was conducted in a BioFlo® 120 bioreactor filled with the same yeast medium, supplemented with sodium acetate 8 g L⁻¹ and antibiotics. The washed cell pellet was inoculated into the medium to a starting OD₆₀₀ of 0.3. The culture was maintained at 30°C, the filtered air supplied at 0.5 SLPM, and the pH was controlled at 7.0 by the automatic titration of 0.5 M HCl. After about 24 hours of batch-mode operation to accumulate biomass, *S. ovata* medium was pumped into the *E. coli* bioreactor (at a working volume of 600 mL) to commence continuous operation. Biomass, acetate, and butanol quantification followed the methods described in Section 2.5 and **Notes S1–S2**.

2.7. Integration of two continuous stirred-tank bioreactors

In the integrated continuous system, Bioreactor1 (BR1) was supplied with fresh anaerobic medium through a connected supply bottle, while Bioreactor2 (BR2) received effluent flow from BR1 at a flow rate of 0.031 mL min⁻¹, and an additional input line supplying L-arabinose and antibiotics (ampicillin, chloramphenicol, and kanamycin) at a flow rate of 0.006 mL min⁻¹. L-arabinose was used as the inducer. The operational parameters of BR1 were identical to those described in Section 2.3. Specifically, BR1 had a working liquid volume of 600 mL and a headspace volume of approximately 400 mL. The gas mixture (H₂/CO₂ = 80:20 at 6 L h⁻¹ and N₂ at 18 L h⁻¹) was continuously fed through a ring sparger at the bottom of BR1. The off-gas exited through a

condenser and a sterile 0.22- μ m filter that was vented to the atmosphere. The pH was controlled at 6.8 by automatic titration with 5 M anoxic NaOH, and the temperature was maintained at 34°C. BR2 had working volumes of 600 mL. The effluent discharged from BR2 was directed to a separate effluent collection bottle. All fluid connections were installed using tubes of the same inner diameter to ensure a constant flow rate.

3. Results and discussion

3.1. Establishing a continuous process for acetate production from CO₂

To establish a stable and continuous supply of acetate for two-stage butanol production, we developed an autotrophic chemostat that enables sustained acetate biosynthesis and direct feeding to the downstream chemostat, overcoming the operational limitations inherent in batch cultivation systems. Before inoculation, *S. ovata* was anaerobically precultured at 34°C, harvested and washed under strict anaerobic conditions, and subsequently transferred into the chemostat. The chemostat was continuously supplied with a gas mixture of 80% H₂ (electron donor) and 20% CO₂ (carbon source) at a flow rate of 6 L h⁻¹, while maintaining a pH of 6.8 and a temperature of 34°C, and an agitation rate of 600 rpm. Under these operating conditions, the volumetric mass transfer coefficient for CO₂ was estimated to be 10.2 h⁻¹ using a dynamic gassing-out approach (**Note S3**). To initiate exponential growth, the process began in batch-mode gas fermentation, during which a specific growth rate of 0.041 ± 0.004 h⁻¹ and a maximum acetate production rate of 1.8 ± 0.2 g L⁻¹ h⁻¹, a CO₂ fixation rate of 1.07 ± 0.04 g L⁻¹ h⁻¹, and a CO₂ conversion rate of $29.6 \pm 1.2\%$ were achieved (see batch operation in **Fig. 2 and Note S4**). The fraction of CO₂ available to the culture was calculated to be 18% (**Note S5**).

Before the culture entered the stationary phase, the operation was switched to continuous mode. Based on the measured growth rate, the dilution rate was set to 0.031 h^{-1} by simultaneously supplying fresh medium and removing culture broth at a volumetric flow rate of 0.31 mL min^{-1} , maintaining a constant volume of 600 mL. Under these conditions the system reached steady state with dry cell weight (DCW) stabilizing at $479 \pm 17\text{ mg L}^{-1}$ and acetate concentration at $13.5 \pm 0.8\text{ g L}^{-1}$ (see continuous operation in **Fig. 2**). Productivities for biomass and acetate were calculated to be $14.8 \pm 0.5\text{ mg L}^{-1}\text{ h}^{-1}$ and $420 \pm 25\text{ mg L}^{-1}\text{ h}^{-1}$, respectively. In addition, CO_2 fixation rate and CO_2 conversion rate were $0.63 \pm 0.04\text{ g L}^{-1}\text{ h}^{-1}$ and $17.8 \pm 1.1\%$ (**Note S4**). The acetogenic chemostat demonstrated high gas-specific productivity and utilization efficiency (**Table 1**), a critical advantage for large-scale applications where reducing gas supply costs is essential for economic viability.

3.2. Metabolic engineering of *E. coli* for butanol production from acetate

With a stable acetate supply base established, the research focus shifted to developing a high-performance *E. coli* strain capable of efficiently converting acetate to butanol. Most previous efforts to produce butanol have centered on glucose-utilizing pathways in engineered *E. coli* under batch or fed-batch operation (Atsumi et al., 2008; Bond-Watts et al., 2011; Davis et al., 2023; Shen et al., 2011). To accommodate acetate as the carbon source, we engineered the biosynthesis of butanol from acetyl-CoA, the central metabolite of acetate metabolism (**Fig. 3A**), with the various strains listed in **Table 2**. All plasmids and primers used are detailed in **Tables S1-S2**. The butanol biosynthesis pathway began with the condensation of two acetyl-CoA molecules to acetoacetyl-CoA catalyzed by acetyltransferase (PhaA). This intermediate was then reduced to 3-

hydroxybutyryl-CoA by a reductase (PhaB). Two enzyme combinations were then evaluated for converting 3-hydroxybutyryl-CoA to crotonyl-CoA and subsequently to butyryl-CoA. The first was crotonase (Crt) and crotonyl-CoA reductase (Ccr), and the second was PhaJ and trans-enoyl-CoA reductase (Ter). Given that the expression efficiency of heterologous genes is influenced by host codon usage bias, both *crt/ccr* and *phaJ/ter* were codon-optimized to GC contents of 50%, matching the native GC content of the host, and to 66%, reflecting the high GC content of the source organism such as *C. necator*, for a comparative analysis (Strains BW1-BW4 in **Table 2**) (Bond-Watts et al., 2011; Gustafsson et al., 2004; Kane, 1995). The final reduction step from butyryl-CoA to butanol was catalyzed by butyraldehyde/butanol dehydrogenase (AdhE2) from *C. acetobutylicum*, which has been reported to exhibit higher activity in *E. coli* compared to AdhE1 (**Fig. 3A**) (Inui et al., 2008). Among the BW1-BW4 strains, only strain BW4 carrying *phaJ/ter* with 50% GC content produced butanol, reaching 111 ± 5 mg L⁻¹ after 48 hours as shown in **Fig. 3B**. BW1, BW2, and BW3 strains showed no detectable butanol production. This result demonstrated that butanol biosynthesis in *E. coli* requires the careful selection of enzymes from suitable microbial sources, combined with effective codon optimization.

To further improve butanol production in strain BW4, we next focused on enhancing acetate assimilation. The biosynthesis of high-value chemicals from acetate typically suffers from low yields and requires metabolic engineering to enhance acetate utilization (Gu et al., 2024; Lee et al., 2018). To overcome this limitation, we overexpressed acetate-assimilating pathways into BW4 to strengthen the supply of acetyl-CoA. Genes encoding acetate kinase–phosphotransacetylase (*ackA-pta*) (Enjalbert et al., 2017; Ricci et al., 2025; Tashiro et al., 2015) and acetyl-CoA synthetase (*acs*) (Gu et al., 2023; Song et al., 2018; Xu et al., 2018), along with a high-activity

mutant *acs* variant (*acsL64IP*) (Novak et al., 2018), were introduced into BW4, generating strains BW5, BW6, and BW7 (**Table 2**). Upon cultivation with 8 g L⁻¹ acetate as a carbon source, strain BW5 proved to be the most effective, producing 341 ± 63 mg L⁻¹ of butanol, whereas BW6 and BW7 produced 133 ± 11 mg L⁻¹ and 133 ± 8 mg L⁻¹, respectively. The observed improvement can be attributed to the energetic efficiency of the *ackA-pta* pathway, which consumes less ATP under microaerobic environments. To further enhance butanol production, we strengthened the *ackA-pta* expression by replacing its arabinose-inducible promoter with a strong constitutive promoter (J23100), constructing strain BW5e (**Table 2**). This optimization step increased butanol production to 371 ± 28 mg L⁻¹. Taken together, this two-step engineering strategy enhanced butanol production by 3.3-fold compared to our starting production strain, BW4.

3.3. Fine-tuning enzyme levels and redox balance for butanol production in shaken bottles

Microbial butanol production is subject to several limitations, including poor acetate assimilation, improper redox balancing, and low efficiency of multigene expression in *E. coli* (Zheng et al., 2009). As shown in **Fig. S2**, butanol biosynthesis theoretically requires four molecules of NADH per molecule of butanol, suggesting that under microaerobic conditions, insufficient NADH regeneration can create a redox imbalance (Liu et al., 2015b; Wen & Shen, 2016). To address this limitation, a *fdh* gene from *C. boidinii* was introduced into either a moderate-copy number plasmid (pCDF-duet vector) or a high-copy number plasmid (pET-duet vector), generating BW5b and BW5c (**Table 2**). FDH catalyzes the oxidation of formate to CO₂ while simultaneously reducing NAD⁺ to NADH (Sharma et al., 2022). This reaction provides an additional intracellular source of NADH, contributing to the redox balance required for butanol biosynthesis. Enhancing NADH

regeneration through FDH expression is expected to help relieve the redox bottleneck under microaerobic conditions and improve butanol yield.

In cultures supplied with 8 g L⁻¹ acetate as the carbon source, BW5b achieved a butanol titer of 385 ± 21 mg L⁻¹, an increase compared to the parental BW5, which produced 341 ± 63 mg L⁻¹ of butanol. In contrast, BW5c gave a butanol titer of 356 ± 51 mg L⁻¹, similar to that of BW5 (**Fig. 3B**). In contrast, BW5g, which was constructed by introducing pCDF-*fdh* into BW5e, produced 304 ± 30 mg L⁻¹ of butanol, a significant decrease from the 371 ± 28 mg L⁻¹ maximum titer of BW5e. These results support the concept of enzyme balance, whereby the limited cellular capacity for recombinant protein expression can be overburdened (Karim & Jewett, 2016), reducing the expression of other essential enzymes and leading to suboptimal formation of the desired product.

To rectify the potential redox imbalance, a transhydrogenase (*pntAB*) known to enhance NADH availability by converting NADPH to NADH was introduced into BW5b and BW5g, generating strains BW5h and BW5j, respectively. BW5j achieved the highest titer of 422 ± 4 mg L⁻¹. The cumulative impact of our metabolic engineering strategy was assessed by performing a time-course comparison of key engineered strains (**Fig. 3C**). The optimized titer represents a 2.1-fold increase over the previously reported titer of 198 mg L⁻¹ for butanol produced from solar-derived acetate (Liu et al., 2015a), demonstrating a significant improvement in productivity.

3.4. Integration of BR1-BR2 continuous bioreactor system for CO₂-to-Butanol production

Based on the optimized strains and butanol production at the 125-mL serum bottle scale, we evaluated engineered *E. coli* strains in a single bioreactor (BR2) to validate their performance for scale-up in the complete two-stage system. This step is crucial, as bottle-scale productivity does

not always translate to bioreactor systems, a challenge previously encountered in similar bioprocesses (Novak et al., 2020). To determine the suitability of engineered *E. coli* strains for continuous operation, batch-mode cultivation of engineered *E. coli* was first conducted in BR2 using *S. ovata* yeast medium supplemented with 8 g L⁻¹ sodium acetate at 34°C, 600 rpm. Upon reaching exponential phase, continuous operation was initiated by feeding fresh medium without integration with BR1. While BW5j was the leading strain at the bottle scale, its performance diminished substantially in the bioreactor. In contrast, strain BW5, while less productive in serum bottles, demonstrated more robust and reliable performance (data not shown). Therefore, BW5 was selected for the subsequent two-stage integrated operation due to its more reliable performance on a larger scale.

Next, we established a two-stage continuous process for butanol production from CO₂ (**Fig. S3**). In this system the first-stage bioreactor (BR1) contains *S. ovata*, which produces acetate from an 80:20 (v/v) H₂/CO₂ gas mixture at a flow rate of 6 L h⁻¹. The acetate-rich culture broth is continuously supplied into the second-stage bioreactor (BR2) where the engineered *E. coli* strain BW5 converts acetate into butanol. BR1 and BR2 were initially operated in batch mode. In BR1, the batch cultivation of *S. ovata* resulted in a specific growth rate of 0.045 h⁻¹, with acetate production increasing in proportion to cell biomass (**Fig. 4A**). In parallel, to ensure sufficient cell mass for BR2, the engineered *E. coli* strain BW5 was cultivated in a medium containing 8 g L⁻¹ sodium acetate at 34°C, 600 rpm and an air flow rate of 0.5 SLPM (Standard Liters Per Minute). When both microorganisms reached their respective exponential growth phases, system integration was initiated by feeding fresh medium to BR1 and transferring culture broth from BR1 to BR2, both at a dilution rate of 0.023 h⁻¹. After 42 hours, the dilution rate increased to 0.031 h⁻¹ to enhance

productivity, and an additional input line supplying L-arabinose and antibiotics was introduced at a flow rate of $0.006 \text{ mL min}^{-1}$, resulting in a total dilution rate of 0.037 h^{-1} . After 90 hours of operation, the system achieved a steady-state, as shown in **Fig. 4A** (bottom). The residence time ($\tau = 1/D$) was 27 hours after the dilution rate was increased to 0.037 h^{-1} . As shown in **Fig. 4A**, butanol and acetate concentrations remained stable between 46 and 121 hours (75 hours). Thus, BR1 successfully supplied the necessary substrate for the second stage by stably maintaining a cell concentration of 0.5 g L^{-1} and an acetate concentration of 14.3 g L^{-1} , with a productivity of $0.4 \text{ g L}^{-1} \text{ h}^{-1}$. In BR2, a final butanol concentration of 93 mg L^{-1} was reached, with a productivity of $3.4 \text{ mg L}^{-1} \text{ h}^{-1}$, while biomass stabilized at 3.9 g L^{-1} . Our results demonstrate that BR1 provides a strong foundation for CO_2 -to-acetate conversion, fixing $22 \text{ g CO}_2 \text{ L}^{-1}$. The integrated system achieves a net CO_2 fixation of $0.36 \text{ g CO}_2 \text{ L}^{-1} \text{ h}^{-1}$ (**Note S6**), and the carbon flow analysis highlights BR2 as a promising stage for further improvement. This two-stage system represents the first proof-of-concept case of producing butanol from CO_2 as the sole external carbon source through an integrated continuous process. However, despite the successful operation of the integrated system, the butanol titer was far below the maximum obtained at the bottle scale, indicating that the process conditions required further engineering. To evaluate whether plasmid instability contributed to the decline in butanol production during continuous operation, the plasmid retention level was monitored in BR2 throughout operation of the chemostat. Colony forming units (CFU) were plated on selective and non-selective agar at approximately 24-h intervals, and the plasmid retention rate remained at 71–81% during continuous operation, even under conditions where butanol was not detected (data not shown). These results indicate that the relatively low butanol production does not correlate with plasmid loss during continuous cultivation.

We next examined the effect of aeration on BR2 performance, as suboptimal oxygen supply was suspected to limit flux through the butanol pathway. In a subsequent integrated run, the airflow rate in BR2 was reduced from 0.5 to 0.3 SLPM to create a more favorable microaerobic condition. This adjustment was based on the observation that elevated oxygen levels in the bottle setup compromised butanol production efficiency, as evidenced by the lower titers previously observed under more aerobic conditions at bottle scale (**Fig. S4**).

A microaerobic condition is optimal for balancing cell growth with butanol synthesis. It provides oxygen for ATP generation to sustain cell growth while limiting respiration, thereby mitigating the diversion of acetyl-CoA and the depletion of NADH, both of which are essential for butanol production. As shown in **Fig. 4B**, the final butanol titer in BR2 increased to 130 mg L⁻¹ with a productivity of 4.8 mg L⁻¹ h⁻¹, representing 40% and 41% improvements over the initial 0.5 SLPM condition. Although biomass slightly decreased to ~2.5 g L⁻¹, the enhanced butanol yield per biomass indicated improved metabolic efficiency. Furthermore, as shown in **Fig. 4B**, butanol production remained stable between 33 and 94 hours, exceeding multiple residence times and confirming that the observed performance reflects steady-state operation. Carbon recovery in BR2 was 81%, accounting for carbon contained in butanol, biomass, and released CO₂. The unrecovered fraction is likely attributable to deviations in actual biomass composition from the assumed chemical formula (which influences the stoichiometry of CO₂ formation) or reduced biomass yield under stress from butanol production and heterologous gene expression. No significant accumulation of pathway intermediates was observed, as all CoA intermediates were detected only at low μM levels (data not shown). These results demonstrate that oxygen availability is a key factor in scaling up butanol biosynthesis and highlight the importance of optimizing aeration

parameters when translating bottle-scale performance into bioreactor operation.

3.5. Economic outlook

We previously developed an analytical framework that combines first-principles-based modeling with technoeconomic analysis to predict the selling price of butanol generated by an industrial-scale version of the process prototyped in the present study (Adams & Clark, 2024). Notably, the TEA model assumes an industrial-ready antibiotic-free strain architecture (e.g., via chromosomal integration), thereby excluding the antibiotic costs required for bench-scale plasmid maintenance. The analysis found that the cost of H₂ and CO₂ would not foreclose economic feasibility as long as hydrogen could be produced for less than \$1 kg⁻¹ (in line with optimistic projections for green hydrogen generation) and CO₂ could be produced for less than \$105 ton⁻¹ (already achievable with point source carbon capture and in line with optimistic projections for direct air capture). Assuming these exogenous conditions are met, bioprocess performance metrics (e.g., productivity, titer, and yield) become the determinants of economic feasibility. Under the conditions simulated by the model, a butanol selling price of \$3.4 L⁻¹ could be achieved, which is comparable to gasoline prices in the regions of the world where fuel is most expensive. However, the projected butanol selling price based on the data obtained in this study is significantly higher as the experimental system underperforms the model in key metrics. Understanding where the experimental results deviate from the simulated process can help identify areas for improvement prior to commercialization.

The experimental BR1 system agreed reasonably well with its *in-silico* counterpart. The selectivity of acetate versus biomass in BR1, with 96.2% of fixed carbon embodied in acetate rather than in biomass, agreed quite well with the value predicted by the model (94.8%). The

acetate titer and productivity of BR1 in the experimental study (14.3 g L^{-1} ; $0.4 \text{ g L}^{-1} \text{ h}^{-1}$) are somewhat lower than the model predictions (22.7 g L^{-1} ; $1.4 \text{ g L}^{-1} \text{ h}^{-1}$). This moderate discrepancy is likely due to differences in the gas-liquid mass transport properties between the experimental BR1 system and the model. These parameters could be addressed during scale-up of the process.

Unlike the results of BR1, the measured productivity, titer, and yield of butanol in BR2 are all significantly lower than those predicted by the model. For instance, the empirical productivity ($4.8 \text{ mg L}^{-1} \text{ h}^{-1}$) is nearly two orders of magnitude lower than that in the model ($296 \text{ mg L}^{-1} \text{ h}^{-1}$). This can in part be explained by the 2.8-fold lower inlet acetate concentration and the 1.5-fold lower dilution rate in the experimental system compared to the simulated system. The bulk of this disparity, however, can be explained by the difference in butanol selectivity between the model and experimental results. The microbe in BR2 will convert acetate either to the desired end-product butanol or to biomass, and this ratio is critical to the economics of the process. The carbon selectivity toward butanol in the modeled system was assumed to be 80% whereas the highest carbon selectivity achieved in the chemostat was 6.6%. According to the economic analysis, the selling price of butanol is roughly inversely proportional to the carbon selectivity, because any decrease in selectivity will result in a corresponding increase in the amount of substrate required.

Butanol selectivity above 80% in engineered *E. coli* has been demonstrated when using glucose as a carbon source (Shen et al., 2011). It is unsurprising that it is more challenging to achieve high selectivity when acetate is used as a carbon source rather than glucose. Glucose is a fermentable sugar, and when grown on glucose anaerobically *E. coli* naturally produces less biomass, as most of the carbon is diverted toward fermentation products to regenerate NAD^+ .

Growth on acetate as a sole carbon source, however, requires respiration, and therefore the cell's central metabolism will favor oxidative phosphorylation and the TCA cycle over the generation of fermentation products.

Balancing the high NADH demand for butanol synthesis with the NADH-consuming oxidative phosphorylation pathway is the primary challenge of this biological conversion. This balance will need to be achieved prior to industrial deployment of this process, likely through a combination of genetic manipulation to control the cells' internal redox metabolism and bioprocess optimization to control oxygen availability. We emphasize that the economics of this process are limited by the selectivity of butanol rather than the fundamental thermodynamics of converting acetate to butanol. It is conceivable that advanced metabolic engineering strategies can continue to be employed toward engineering *E. coli* for the conversion of acetate to butanol, which will bring this process closer to economic viability.

4. Conclusions

We demonstrated the continuous, two-stage bioprocess capable of converting CO₂ into butanol by combining an autotrophic acetogen with an engineered *E. coli* biocatalyst. A key challenge was engineering an *E. coli* strain to efficiently convert acetate. The combinations of gene modules and redox-balancing strategies resulted in an improved titer at the bottle scale (~422 mg L⁻¹). While the integrated system produced butanol from CO₂ via acetate, the final titer (~130 mg L⁻¹) and productivity remain far below industrially relevant levels, indicating the need for further optimization.

Our analysis reveals that this gap stems from a low carbon selectivity toward butanol, which is far below the ~80% target required for economic viability (Adams & Clark, 2024). Closing this

gap will therefore require a co-optimization strategy that combines advanced metabolic engineering to lock in high-selectivity pathways with dynamic process control. Nonetheless, this interplay between our previous modeling and this study demonstrates the synergy that these two approaches bring to the development of novel bioprocesses.

Ethics declarations (Competing interests)

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that supports the findings of this study are available from the corresponding authors upon reasonable request.

Credit authorship contribution statement

D.S.C., P.Y., J.D.A., H.-J.J., and J. Kim conceptualized and designed the study. H.-J.J., H.-J.C. and J. Kim performed the experiments, conducted the validation and formal analysis, and prepared the initial draft of the manuscript. D.S.C., P.Y., H.-J.J., H.-J.C., and J.D.A. discussed data interpretation. P.Y. and D.S.C. provided resources and supervision, administered the project, secured funding, and contributed to writing the original draft. All authors discussed the results and

478 contributed to the revision of the manuscript.

479

480 **Appendix A. Supplementary materials**

481 Supplementary data for this work can be found in e-version of this paper online.

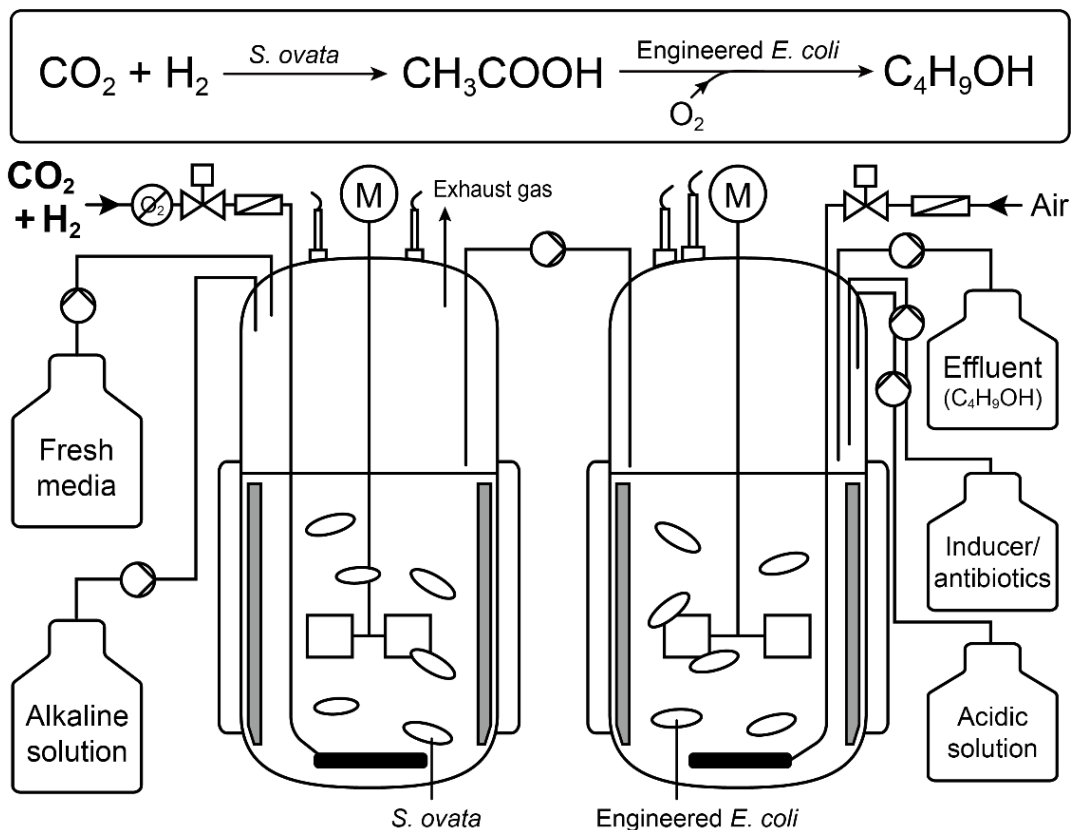


Fig. 1. Schematic of microbial production of butanol with two chemostats. In BR1, CO_2 is converted to acetate by *S. ovata*. The acetate produced in BR1 is subsequently transferred to BR2, where it serves as a carbon source for engineered *E. coli* to synthesize butanol. *S. ovata*: *Sporomusa ovata*. BR1: continuous stirred-tank bioreactor 1 (left); BR2: continuous stirred-tank bioreactor 2 (right).

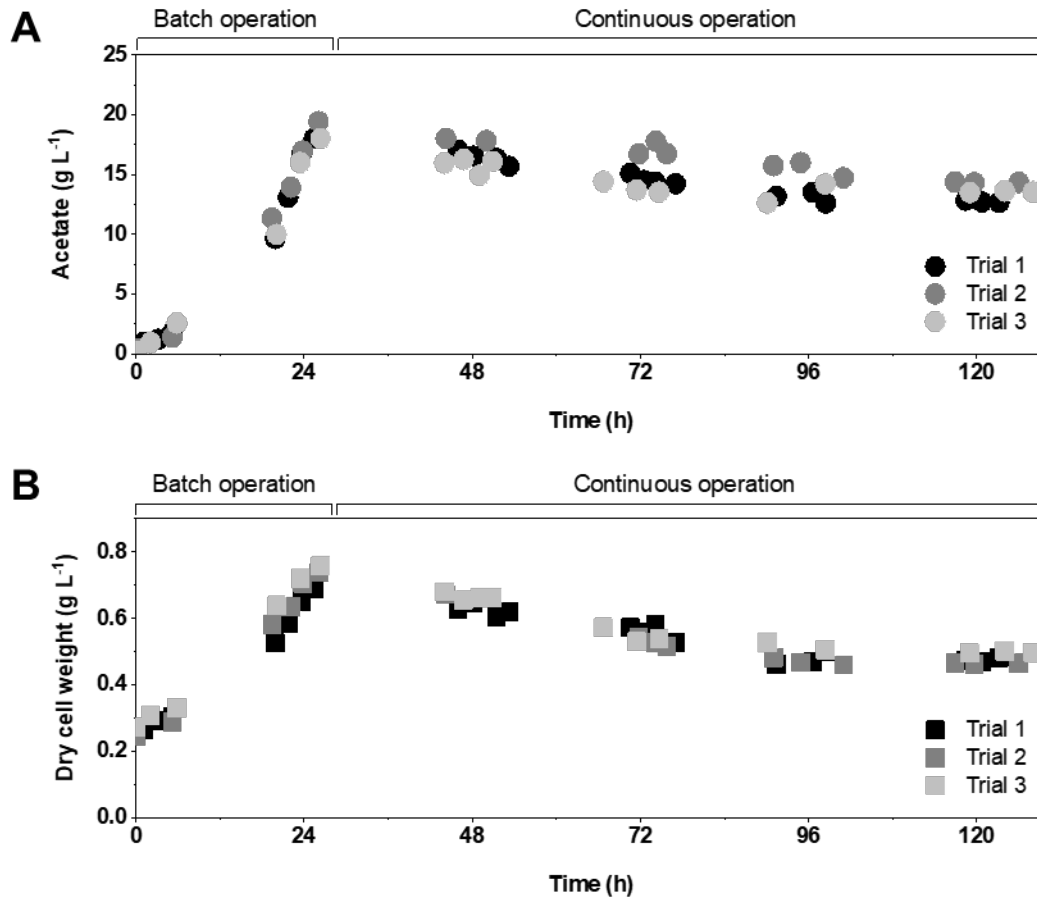


Fig. 2. Steady-state CO₂ conversion using a chemostat under anaerobic conditions. Time profiles of (A) acetate concentration (circles) during batch and continuous operation. The steady-state acetate concentration was $13.5 \pm 0.8 \text{ g L}^{-1}$ and the corresponding productivity was $420 \pm 25 \text{ mg L}^{-1} \text{ h}^{-1}$. (B) Dry cell weight (squares) of *S. ovata* was $479 \pm 17 \text{ mg L}^{-1}$ at steady-state. Dilution rate: 0.031 h^{-1} ; H₂/CO₂ feed rate: 6 L h^{-1} ; temperature: 34°C ; working volume: 600 mL ; agitation: 600 rpm ; pH: 6.8 (maintained through the automated addition of 5 M NaOH).

Table 1. Comparison of chemostat's performance for acetogenic bacteria

Acetogen	Steady-state concentration (g L ⁻¹)	Productivity (mg L ⁻¹ h ⁻¹)	Acetate			Biomass	
			Cell-specific productivity (mg _{acetate} g _{cell} ⁻¹ h ⁻¹)	CO ₂ -specific productivity (mg L _{medium} ⁻¹ L _{CO₂} ⁻¹)	H ₂ -specific productivity (mg L _{medium} ⁻¹ L _{H₂} ⁻¹)	Steady-state dry cell weight (mg L ⁻¹)	Productivity (mg L ⁻¹ h ⁻¹)
<i>S. ovata</i> ^a	13.5 ± 0.8	420 ± 25	878 ± 40	350 ± 20.8	87.5 ± 5.2 ^c	479 ± 17	14.8 ± 0.5
<i>A. woodii</i> ^b	22	758	725 ± 75	148.6 ^c	63.2 ^c	1100	38.5 ^c

^aThis work. ^b*J. Biotechnol.* 212:11-18 (2015) (Kantzow et al., 2015). Reported values represent means ± standard deviation ($n = 3$). ^cCalculation based on data reported in the corresponding reference. Gas-specific productivities were determined by dividing productivity of acetate by the volumetric flow rate of the CO₂ and H₂, respectively. *A. woodii*: *Acetobacterium woodii*.

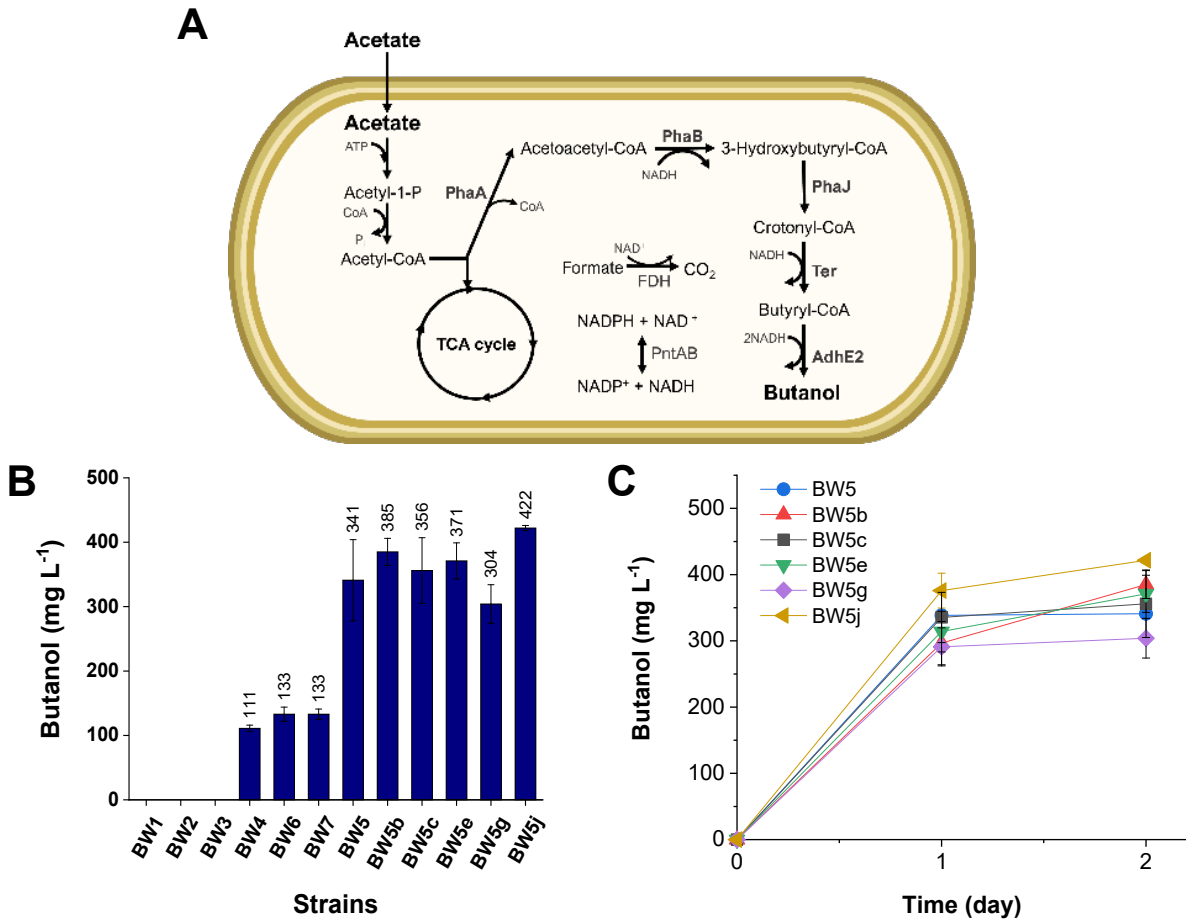


Fig. 3. Metabolic engineering of *E. coli* BW25113 for enhanced butanol production from acetate. (A) Schematic representation of the butanol biosynthetic pathway constructed in *E. coli*. Acetate-derived acetyl-CoA is converted to butanol via PhaA, PhaB, PhaJ, Ter, and AdhE2. Specifically, (1) PhaA from *C. necator* catalyzes the condensation of two acetyl-CoA molecules to acetoacetyl-CoA; (2) PhaB from *C. necator* reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA; (3) crotonase from *C. acetobutylicum* and PhaJ from *A. caviae* convert 3-hydroxybutyryl-CoA to crotonyl-CoA; (4) Ccr from *S. collinus* and Ter from *T. denticola* reduce crotonyl-CoA to butyryl-CoA; and (5) AdhE2 from *C. acetobutylicum* converts butyryl-CoA to butanol (Table S4). (B) Butanol titers (mg L⁻¹) of engineered *E. coli* strains after 48 h cultivation in acetate-containing *S. ovata* yeast medium. Strains carry various combinations of the heterologous pathway genes (Table 2) and were cultivated under identical conditions. Data are presented as mean $\pm$ standard deviation from triplicates. (C) Time-course of butanol production by engineered *E. coli* strains cultivated in *S. ovata* yeast medium supplemented with 8 g L⁻¹ sodium acetate in the bottle-scale. Cultures were incubated at 34°C for up to 48 h, and samples were collected every 24 h. The plotted strains include BW5 (blue circles), BW5b (red triangles), BW5c (black squares), BW5e (green inverted triangles), BW5h (purple diamonds), and BW5k (orange right-pointing triangles). Data are shown as mean $\pm$ standard deviation from triplicates.

Table 2. Summary of engineered *E. coli* strains, their genotypes, and final butanol titers (mg L⁻¹). All experiments were performed in triplicate, and butanol titers are presented as mean ± SD (n = 3).

Label	<i>E. coli</i> Strain	Gene description	Content	Titer (mg L ⁻¹)
BW1	BW25113	<i>phaA, phaB, crt, ccr, adhE2</i>	Codon usage(66%)	N.D.
BW2	BW25113	<i>phaA, phaB, crt, ccr, adhE2</i>	Codon usage(50%)	N.D.
BW3	BW25113	<i>phaA, phaB, phaJ, ter adhE2</i>	Codon usage(66%)	N.D.
BW4	BW25113	<i>phaA, phaB, phaJ, ter adhE2</i>	Codon usage(50%)	111 ± 5
BW5	BW25113	<i>phaA, phaB, phaJ, ter, adhe2, ackA-pta</i>	Acetate assimilation	341 ± 63
BW6	BW25113	<i>phaA, phaB, phaJ, ter, adhE2, acs</i>	Acetate assimilation	133 ± 11
BW7	BW25113	<i>phaA, phaB, phaJ, ter, adhE2, acsL641P</i>	Acetate assimilation	133 ± 8
BW5b	BW25113	<i>phaA, phaB, phaJ, ter, adhe2, ackA-pta, fdh</i>	FDH (moderate-copy)	385 ± 21
BW5c	BW25113	<i>phaA, phaB, phaJ, ter, adhe2, ackA-pta, fdh</i>	FDH (high-copy)	356 ± 51
BW5e	BW25113	<i>phaA, phaB, phaJ, ter, adhE2, ackA-pta (P₁₂₃₁₀₀)</i>	Strong promoter	371 ± 28
BW5g	BW25113	<i>phaA, phaB, phaJ, ter, adhe2, ackA-pta (P₁₂₃₁₀₀), fdh</i>	Strong promoter, FDH	304 ± 30
BW5h	BW25113	<i>phaA, phaB, phaJ, ter, adhE2, ackA-pta (P_{arabinose}), fdh, pntAB</i>	FDH, transhydrogenase	N.D.
BW5j	BW25113	<i>phaA, phaB, phaJ, ter, adhe2, ackA-pta (P₁₂₃₁₀₀), fdh, pntAB</i>	FDH, strong promoter, transhydrogenase	422 ± 4

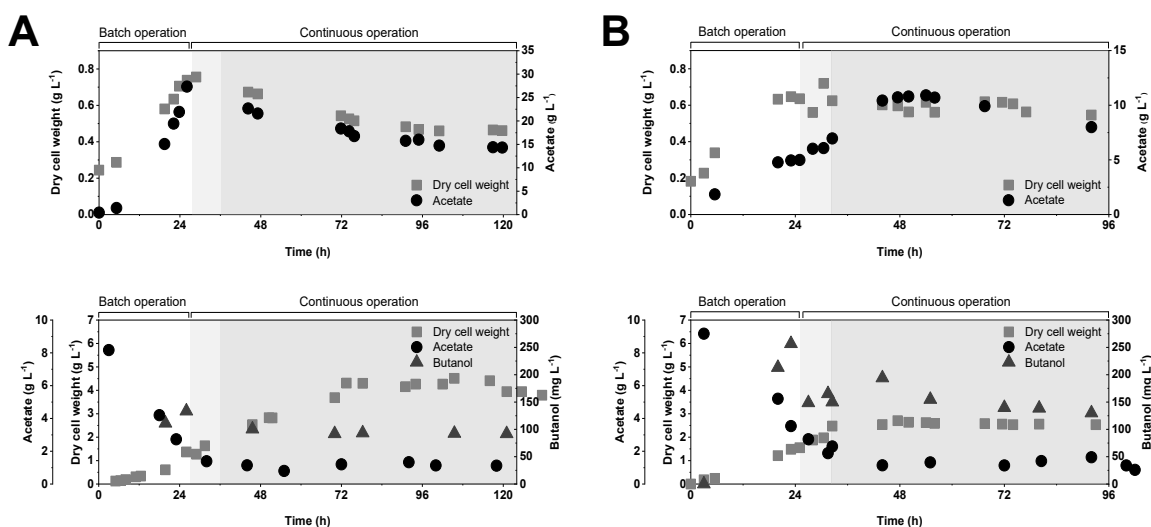


Fig. 4. Biomass, acetate, and butanol production for two separate experiments in BR1 and BR2 under batch and continuous, steady-state operation. (A) Time profiles of BR1 (top) and BR2 (bottom). The batch phase (white background) exhibited an initial growth phase with a specific growth rate of 0.045 h^{-1} . Continuous operation (shaded region) was initiated at a dilution rate of 0.023 h^{-1} followed by an increase to 0.031 h^{-1} . Acetate concentration (black circles) and productivity in BR1 stabilized at $\sim 14.3 \text{ g L}^{-1}$ and $0.44 \text{ g L}^{-1} \text{ h}^{-1}$, while biomass concentration (gray squares, top) stabilized around 0.46 g L^{-1} . BR2 was operated initially at a dilution rate of 0.026 h^{-1} , which was later increased to 0.037 h^{-1} . The butanol titer and productivity stabilized at 93 mg L^{-1} and $3.4 \text{ mg L}^{-1} \text{ h}^{-1}$, while biomass (gray squares, bottom) stabilized at 3.9 g L^{-1} . (B) Time profile of BR1 (top) under the same dilution rates as in (A). Acetate (black circles) concentration and productivity stabilized at $\sim 8.0 \text{ g L}^{-1}$ and $0.3 \text{ g L}^{-1} \text{ h}^{-1}$, while biomass (gray squares) stabilized around 0.55 g L^{-1} . The butanol titer and productivity in BR2 (bottom) reached 130 mg L^{-1} and $4.8 \text{ mg L}^{-1} \text{ h}^{-1}$, while biomass stabilized at 2.5 g L^{-1} .

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