Production of an acetone-butanol-ethanol mixture from *Clostridium acetobutylicum* and its conversion to high-value biofuels

Sanil Sreekumar[1,2], Zachary C Baer[2,3], Anbarasan Pazhamalai[1,2], Gorkem Gunbas[1,2], Adam Grippo[1,2], Harvey W Blanch[2,3], Douglas S Clark[2,3] & F Dean Toste[1,2]

[1]Department of Chemistry, University of California, Berkeley, California, USA. [2]Energy Biosciences Institute, University of California, Berkeley, California, USA. [3]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, California, USA. Correspondence should be addressed to H.W.B. (blanch@berkeley.edu), D.S.C. (clark@berkeley.edu) or F.D.T. (fdtoste@berkeley.edu).

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Clostridium acetobutylicum **is a bacterial species that ferments sugar to a mixture of organic solvents (acetone, butanol and ethanol). This protocol delineates a methodology to combine solventogenic clostridial fermentation and chemical catalysis via extractive fermentation for the production of biofuel blendstocks. Extractive fermentation of *C. acetobutylicum* is operated in fed-batch mode with a concentrated feed solution (500 grams per liter glucose and 50 grams per liter yeast extract) for 60 h, producing in excess of 40 g of solvents (acetone, butanol and ethanol) between the completely immiscible extractant and aqueous phases of the bioreactor. After distillation of the extractant phase, the acetone, butanol and ethanol mixture is upgraded to long-chain ketones over a palladium-hydrotalcite (Pd-HT) catalyst. This reaction is generally carried out in batch with a high-pressure Q-tube for 20 h at 250 °C. Following this protocol enables the production of ~0.5 g of high-value biofuel precursors from a 1.7-g portion of fermentation solvents.**

INTRODUCTION

Acetone-butanol-ethanol (ABE) fermentation of sugar using solventogenic strains of *Clostridium* is a well-known industrial process, and it was used during the early and middle 20th century for the production of solvents. Originally, the desired product of the fermentation was acetone for the production of cordite[1] (a smokeless munitions powder) during the first World War. After the war, there was little need for acetone, but the demand for butanol skyrocketed as a solvent for lacquers, and by 1945 nearly 65% of all butanol was produced by ABE fermentation[2]. It was during this period of history that ABE fermentation ranked second only to ethanol fermentation in scale and importance[3]. However, by the late 1950s, routes to produce solvents from petroleum were made cost-competitive with fermentation[4]. In addition, the major feedstock at the time was molasses, which spiked in price because of animal feed demand. These two factors led to the end of industrial ABE fermentation throughout most of the world.

The growing demand for renewable transportation fuels to mitigate greenhouse gas emissions is being enabled by both the development of new technologies[5–7] and the improvement of once commercially established processes[8,9]. For this reason, ABE fermentation has seen renewed attention for the production of butanol, which has wide applications in the energy and chemical industries. Although China is leading the re-commercialization of ABE fermentation with over 210,000 MT of butanol capacity[10], plans for different stages of preparation and scale-up in Brazil, the US, the UK and France are also underway. However, for ABE to be a commercially viable process, high butanol yields and titers are required; this is generally achieved by engineering strains of *Clostridium*. Recently, with the aid of genetic, metabolic and protein engineering, several groups have demonstrated strategies to selectively improve butanol production in solventogenic *Clostridium*[11–13]. However, even with these modest improvements, the ABE process remains economically viable for the specialty chemical market alone. The physical properties of acetone, butanol and ethanol do not align with traditional jet and diesel fuels, specifically energy density and volatility. In addition, both ethanol and butanol form azeotropes with water, and thus they require multiple distillation columns and a substantial amount of energy to purify each solvent separately.

To circumvent the need for these separation steps and to expand the portfolio of available biofuel processes, we recently described a catalytic strategy to upgrade all three of the solvents produced during ABE fermentation to long-chain ketones, which after hydrodeoxygenation produce the types of hydrocarbons that are components of gasoline, jet and diesel fuel[14]. We adopted a two-step approach wherein sugars were first catabolized to acetone, butanol and ethanol using *C. acetobutylicum*. The overall solvent productivity and titer of this fermentation was significantly improved by using *in situ* solvent removal with the water-immiscible nontoxic extractant glyceryl tributyrate. Continuous extractive fermentation to remove butanol from *C. acetobutylicum* cultures showed similar results over much longer fermentation times[15]. The mixture of fermentation products was then catalytically upgraded over Pd/C-K_3PO_4 to hydrocarbon ketones in excellent overall yield. Subsequently, recyclability of the catalyst was improved by using multifunctional hydrotalcite, which provided support for the metal in dehydrogenation of alcohols and served as the base in the condensation reaction[16]. Methods to rapidly compare nontoxic extractants for efficient removal of fermentation products *in silico* have been previously described[16]. By combining the fields of catalysis and fermentation, this protocol is fundamentally different from all previously described routes to produce biofuels.

Although we have included this as a single procedure, we envision that there are research applications in which people might want to only perform the extractive fermentation or perform the catalytic reaction using commercially derived solvents.

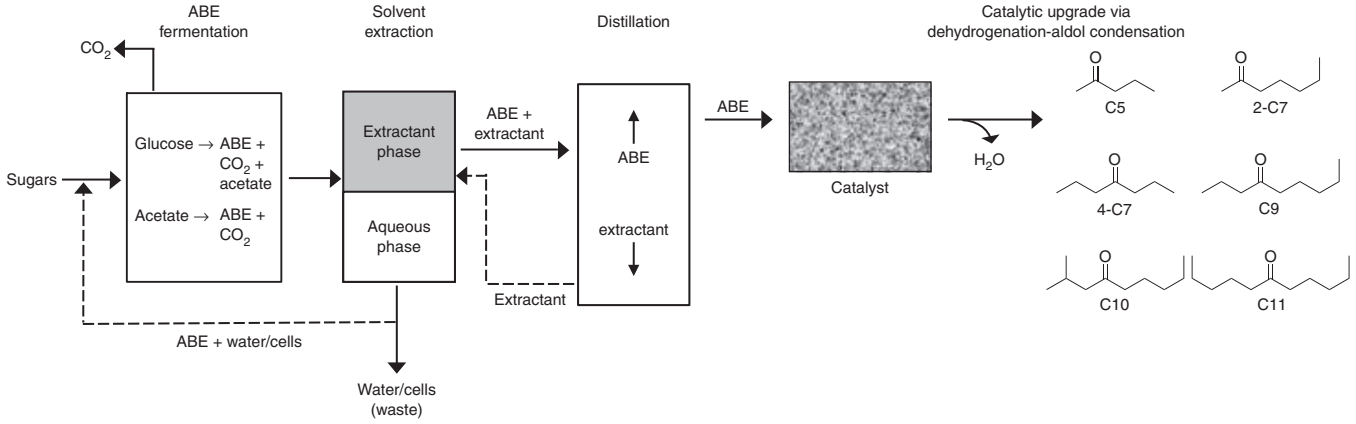


Figure 1 | Process flow diagram for extractive fermentation coupled with heterogeneous catalysis to produce biofuel blendstocks. Dashed lines show processes for recycling reagents.

More specifically, we previously showed that engineering *C. acetobutylicum* to produce isopropanol, butanol and ethanol or to alter the ratio of butanol:ethanol improved the catalytic production of higher MW diesel precursors[13,16]. Furthermore, the concept of an integrated fermentation and catalysis process is not limited to only solventogenic *Clostridium* fermentation and alkylation reactions, with several promising biochemical processes recently described[17].

The protocol described here allows experimenters to readily produce blends of ketones from glucose or sucrose via an extractive fermentation coupled with a heterogeneous catalytic reaction; see **Figure 1**. After this protocol, the biological fermentation and chemical catalysis can be carried out on kilogram and gram scale, respectively; further scale-up requires advanced separation and distillation unit operations. This reaction scale produces sufficient blendstock precursors for fuel testing.

MATERIALS

REAGENTS

! CAUTION All chemicals in this protocol should be handled with care using standard safety equipment (eye protection, gloves, laboratory coat, ventilated fume hood and so on).

- *C. acetobutylicum* (American Type Culture Collection (ATCC) 824; see **Box 1** and **Fig. 2** for preculture procedure)
- D-(+)-Glucose (Sigma-Aldrich, cat. no. G8270)
- Bacto yeast extract (BD, cat. no. 212750)
- Ammonium acetate (Sigma-Aldrich, cat. no. 25006)
- NaCl (Fisher Scientific, cat. no. BP358)
- KH_2PO_4 (Fisher Scientific, cat. no. P285)
- K_2HPO_4 (Fisher Scientific, cat. no. BP363)
- KOH pellets (Sigma-Aldrich, cat. no. 221473)
- Cysteine HCl·H_2O (Amresco, cat. no. 0206)
- $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, cat. no. M63)
- $MnSO_4 \cdot H_2O$ (Acros Organics, cat. no. 423915000)
- $FeSO_4 \cdot 7H_2O$ (Spectrum Chemicals, cat. no. F1060)
- Antifoam 204 (Sigma-Aldrich, cat. no. A8311)
- Tributyrin, 98% (Acros Organics, cat. no. 15088010)
- Phosphoric acid, 85% (Sigma-Aldrich, cat. no. 345245)
- Ethyl alcohol, 200 proof (Sigma-Aldrich, cat. no. 459844)
- Sulfuric acid solution, 5 M (Sigma-Aldrich, cat. no. 35347)
- Ethyl acetate, anhydrous, 99.8% (Sigma-Aldrich, cat. no. 270989)

Synthesis

- Palladium(II) nitrate dihydrate (Sigma-Aldrich, cat. no. 76070)
- Copper(II) nitrate hemi(pentahydrate) (Sigma-Aldrich, cat. no. 12837)
- Hydrotalcite, synthetic (Sigma-Aldrich, cat. no. 652288)
- Acetone, ≥99.9% (Sigma-Aldrich, cat. no. 650501)
- 1-Butanol, ≥99.7% (Sigma-Aldrich, cat. no. 34867)
- Ethanol, ≥99.5% (Sigma-Aldrich, cat. no. 459844)
- Dodecane, ≥99% (Sigma-Aldrich, cat. no. 297879)
- Tetrahydrofuran, ≥99.9%, inhibitor-free (Sigma-Aldrich, cat. no. 401757)

EQUIPMENT

▲ CRITICAL The defining piece of equipment for this protocol is the Bioreactor. You could choose any instrument that is capable of controlling agitation, pH and temperature; sparging sterile nitrogen gas and chilling off-gas back to the bioreactor. In our laboratory, we use the 3.5-liter (2.5-liter working volume) RALF bioreactor (Bioengineering); the required components are listed below. See **Box 2** for preparation steps that need to be performed before starting the main PROCEDURE.

Bioreactor components

- pH probe 405-DPAS-SC-K8S (Mettler Toledo)
- InPro6900 dissolved oxygen (DO) probe (Mettler Toledo)
- Temperature probe (Mettler Toledo)
- Filters, 0.22 µm (autoclavable) for nitrogen input and exhaust gases
- Cooling condenser
- Media sampling line with Luer lock fitting and cap
- Water bath (optional)—needed if the bioreactor system does not compensate
- Extractant sampling line with Luer lock fitting and cap—end kept 2 inches above the final medium volume line
- Concentrated medium addition line with Luer lock fitting and cap
- Base feed line with Luer lock fitting and cap
- Silicone tubing—tubing of two sizes needed: one with an inner diameter (i.d.) of 1.6 mm and outer diameter (o.d.) of 4.8 mm and the other with an i.d. of 4.8 mm and an o.d. of 7.9 mm
- Pumpable base reservoir bottle
- Peristaltic pump and pump head—capable of pumping the tubing with a 7.9-mm o.d. at a volumetric flow rate in excess of 50 ml min^{-1}
- Thermo RTE 7 115V/60Hz chiller with a Digital Plus controller—chiller bath with ethylene glycol capable of holding at 2–4 °C

Analytical equipment

- Agilent HPLC with Aminex HPX-87H ion exchange column, refractive index and UV/visible detectors

Box 1 | Bacteria inoculation and preculture ● TIMING 1 h (plus 24 h of incubation time)

1. Thaw 0.5 ml of frozen anaerobic glycerol stock of *C. acetobutylicum* ATCC 824 and inject it aseptically with a sterile 1-ml syringe and 22-G1 needle into a Hungate tube containing 10 ml of anaerobic CGM.
! CAUTION Use appropriate safety precautions when handling needles.
2. Incubate the culture at 37 °C for 12–20 h to an OD_{600} of 1–2.0.
3. Using a sterile 5-ml syringe and 22G1 needle, aseptically transfer 4 ml of the overnight culture to 100 ml of fresh CGM in an anaerobic jar.
! CAUTION Use appropriate safety precautions when handling needles.
4. Incubate the 100-ml preculture at 37 °C for 4–6 h to an OD_{600} of 1–2.0. To measure the OD_{600} on a cuvette spectrophotometer, place the instrument in absorbance mode, set the absorbance wavelength to 600 nm and blank the instrument with a cuvette containing 1 ml of deionized water. In a separate cuvette, add 1 ml of the preculture and measure the absorbance value.
▲ CRITICAL STEP If the absorbance at 600 nm reads >0.5, dilute it with deionized water. Calculate actual OD_{600} by multiplying (absorbance value) × (dilution factor). Doubling time for ATCC 824 at 37 °C is ~1 h during exponential growth. A representative plot and table of OD_{600} values for the fermentation is provided in **Figure 4** in the 'ANTICIPATED RESULTS' section.

- Varian gas chromatograph with FactorFour column, VF-5ms (Varian, cat. no. CP8944) and flame-ionization detector (FID)
- Cuvette spectrophotometer—any spectrophotometer can be used as long as it can measure absorbance at 600 nm
- Benchtop centrifuge capable of at least 5,000*g*
- YSI Biochemistry Analyzer 2300 with glucose membrane (part no. 2365)

Synthesis
- Q-tube, 12 ml (Q-labtech)
- Q-parallel synthesizer (Q-labtech)
- Blue butyl rubber septum stoppers, 20 mm (Bellco Glass, cat. no. 2048-11800A)
- Teflon-coated magnetic stir bar
- Large safety shield (Scienceware, cat. no. S51223)
- Magnetic hotplate stirrer
- Porcelain mortar and pestle (100-mm diameter; 100-ml capacity)
- Weighing balance
- Weighing paper
- Weighing boats
- Medium frit filter funnel, lower vacuum assembly (Chemglass; 60 ml, 24/40 joint)
- Erlenmeyer filtering flask (Chemglass; 250 ml)
- Graduated cylinder (Vitlab; 250 ml)
- Yellow Teflon caps
- Disposable syringes (1, 5, 10 and 20 ml)
- Disposable needles (18, 20 and 22 gauge; 2, 4 and 6 inches long)
- Metal spatula
- Pasteur pipettes
- Rotary evaporator
- Vacuum pump
- Vacuum manifold with vacuum line
- Rubber vacuum tubes
- Reflux condenser
- Varian gas chromatograph with flame-ionization detector (FID)
- FactorFour column VF-5ms (Varian, cat. no. CP8944)
- Centrifuge capable of at least 5,000*g*

Inoculum preparation
- Hungate tube capable of being autoclaved, sealed and maintained anaerobic
- Anaerobic jar capable of being autoclaved, sealed and capable of holding 100 ml of liquid
- Blue butyl stoppers, 20 mm, must be impervious to oxygen and fit Hungate tube and anaerobic jar
- Aluminum crimp seals, 20 mm, capable of keeping butyl stopper in place during sealed autoclave
- Seal crimper—for 20-mm aluminum seals
- Syringes—1 and 5 ml (sterile)
- Needles—22-G1 sterile
- Incubator capable of maintaining a temperature of 37 ± 2 °C

REAGENT SETUP

Metals solution 100 ml of metals solution contains 5 grams per liter $MgSO_4{\cdot}7H_2O$, 0.5 grams per liter $MnSO_4{\cdot}H_2O$ and 0.5 grams per liter $FeSO_4{\cdot}7H_2O$. Filter-sterilize the solution through a 0.22-µm filter. The expected shelf life at room temperature (25 °C) is 1 week.

***Clostridium* growth medium** 110 ml of *Clostridium* growth medium (CGM) contains 70 grams per liter glucose, 5 grams per liter yeast extract, 2 grams per liter ammonium acetate, 1 gram per liter NaCl, 0.75 grams per liter KH_2PO_4, 0.75 grams per liter K_2HPO_4, 0.5 grams per liter cysteine $HCl{\cdot}H_2O$ and 220 µl of metals solution. Sparge the medium with N_2 for 30 min, and then dispense 10 ml into the Hungate tube and 100 ml into the anaerobic serum bottle. Seal the tube and the bottle with a butyl stopper and crimp-on aluminum cap. Sterilize the medium by autoclaving for 20 min on liquid cycle. **! CAUTION** Use standard precautions when handling pressurized glassware after autoclaving the sealed Hungate tubes and serum bottles. The expected shelf life at room temperature is 2 weeks. **▲ CRITICAL** Glucose can be substituted with sucrose as the primary carbon source, but this leads to much longer fermentation times before strong ABE production is observed.

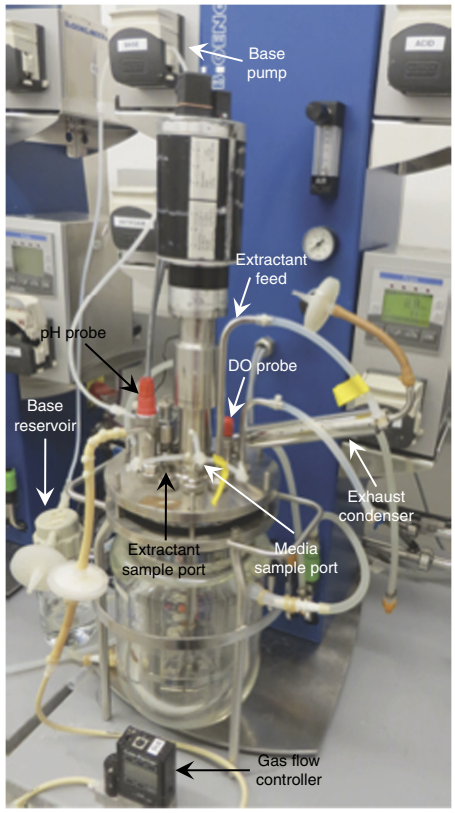


Figure 2 | Assembled extractive fermentation bioreactor.

Box 2 | Fermentor setup ● TIMING 2 h (plus 6 h of incubation time, 3 h of N_2 sparging)

Setup is depicted in **Figures 2** and **3**.

1. Immerse the pH probe in pH 7.0 solution for at least 20 min or until the probe output signal stabilizes. Calibrate the probe to pH 7.0. Rinse the probe with water and repeat this in pH 4.0 solution.

▲ CRITICAL STEP pH calibration is sensitive to temperature; if your bioreactor system does not compensate for temperature effects then keep the pH 7.0 and 4.0 solutions in a water bath held at 37 °C while performing the pH calibration.

2. Thoroughly rinse the bioreactor vessel, ports and lines with deionized water to remove any residual material from previous experiments.

3. Add 750 ml of deionized water to the bioreactor; insert the calibrated pH probe, DO probe and temperature probe. Cap all sampling, feed, acid and base ports, and place 0.22-µm filters on the N_2 supply and cooling condenser exhaust. Cover filters with aluminum foil to prevent the filter from flooding with water. Autoclave the bioreactor for 30 min on liquid cycle.

▲ CRITICAL STEP The DO probe requires 6 h to polarize post autoclaving.

4. Once the bioreactor has cooled, add 200 ml of growth medium and initiate N_2 sparging at 200 ml min^{-1} to make the bioreactor anaerobic. Set the bioreactor temperature to 37 ± 0.2 °C and agitation to 200 r.p.m. Allow N_2 sparging for 3 h before the bioreactor is considered anaerobic. Prime the pump line connecting the 5 M KOH base reservoir and fermentor.

Ethanol, 70% (vol/vol) Add 350 ml of 200 proof ethyl alcohol to 150 ml of sterile water. This solution is used for sterilizing Luer lock connections and caps on the bioreactor, and it can be prepared and stored at room temperature for up to 10 d before fermentation. Store it at room temperature for up to 1 month.

Bioreactor growth medium 200 ml of medium contains 350 grams per liter glucose, 25 grams per liter yeast extract, 10 grams per liter ammonium acetate, 5 grams per liter NaCl, 4.25 grams per liter KH_2PO_4, 4.25 grams per liter K_2HPO_4, 2.5 grams per liter cysteine HCl·H_2O, 2 ml of metals solution and 100 µl of Antifoam 204. Filter-sterilize the solution through a 0.22-µm filter and pour it into a sterile reservoir bottle. ▲ CRITICAL Use the same sugar species in the bioreactor growth medium as prepared for the Hungate tube and anaerobic serum bottle. Freshly prepare the solution for each experiment.

Concentrated *Clostridium* medium 500 ml of concentrated *Clostridium* medium contains 450 grams per liter glucose and 50 grams per liter yeast extract. Heat the solution in a 65 °C water bath to completely dissolve the solids. Filter-sterilize the medium with a 0.22-µm filter. Prepare the medium on the day of fermentation. ▲ CRITICAL The estimated time to completely dissolve the sugar and yeast extract is 2 h at 65 °C. At room temperature, complete dissolution can take up to 8 h. Freshly prepare the medium for each experiment.

Acid and base solutions Prepare 200 ml of 5 M KOH by carefully dissolving 56.1 g of KOH pellets in 120 ml of deionized water. After the pellets have completely dissolved, adjust the final volume to 200 ml with deionized water. ! CAUTION The solution will become very hot as KOH dissolves. Prepare the solution in a chemical hood and wait for the solution to return to room temperature before handling. Place the solution in a pumpable reservoir bottle with a 0.22-µm filtered vent line. Prepare 10 ml of 20 wt% phosphoric acid in water. ! CAUTION Use standard precautions when handling concentrated acid. Store it in an appropriate corrosives cabinet at room temperature for up to 3 months.

HPLC standards Accurately weigh out 4.00 g of sugar (glucose or sucrose), 1.00 g of glacial acetic acid, 1.26 g of sodium butyrate (1 g eq. butyric acid), 1.26 g of sodium lactate (1 g eq. lactic acid), 1.50 g of acetone, 1.50 g of 200 proof ethyl alcohol and 1.50 g of *n*-butanol into 80 ml of sterile water. Adjust the final volume to 100 ml in a volumetric flask. Dilute the sample 2×, 4×, 10×, 20× and 50× with deionized water. Freshly prepare the standards with each experiment.

Gas chromatography (GC) standards Accurately weigh out 2.0 g of acetone, 2.0 g of 200 proof ethyl alcohol and 4.0 g of *n*-butanol into 80 ml of tributyrin. Adjust the final volume to 100 ml in a volumetric flask. Dilute the sample 2×, 4×, 10×, 20× and 50× with tributyrin. Freshly prepare the standards with each experiment.

EQUIPMENT SETUP

Fermentor This is set up as described in **Box 2** and **Figure 3**.

HPLC analysis of fermentation broth This HPLC method allows for quantification of sugar (glucose or sucrose), acetic acid, butyric acid, lactic acid, acetone, ethanol and butanol. Calibration curves for these compounds should be prepared periodically. In our laboratory, we use an Agilent HPLC with UV-visible (measurement of acetone and butyric acid) and refractive index (detection of all other compounds) detectors, and analysis is performed using an Aminex HPX-87H ion exchange column. The settings used are summarized below:

Mobile phase	0.01 NH_2SO_4 in deionized water
Flow rate	0.7 ml min^{-1}
Temperature	35 °C
Injection volume	20 µl (10 µl for initial time point)
Acetone UV/visible detection wavelength	265 nm
Butyric acid UV/visible detection wavelength	190 nm

GC analysis of extractant phase This GC method allows for the quantification of acetone, ethanol and butanol. Calibration curves for these compounds should be prepared periodically. In our laboratory, we use a Varian gas chromatograph with a FactorFour column VF-5ms and FID.

Injector wash fluid	Ethyl acetate
Injector temperature	260 °C
Detector temperature	280 °C
Injection volume	1 µl
Split ratio	1:60
Air flow	300 ml min^{-1}
Detector H_2 flow	30 ml min^{-1}
Detector makeup flow	25 ml min^{-1}
Oven temperature profile	35 °C hold for 3 min Ramp to 150 °C at 10 °C min^{-1} Ramp to 300 °C at 20 °C min^{-1} 300 °C hold for 7 min

PROCEDURE

Batch-phase fermentation ● TIMING 30 min (plus 18 h of incubation time)

1| Adjust the bioreactor pH to 5.8 with a 20 wt% phosphoric acid solution (~1–3 ml) before inoculating cells. Use a 5-ml syringe through the acid port of the bioreactor.

2| With a 60-ml syringe and 18G1 needle, aseptically transfer 50 ml of *Clostridium* culture into the 950-ml anaerobic bioreactor. A starting OD_{600} of 0.05–0.1 should be measured.
! CAUTION Use appropriate safety precautions when handling needles.

3| After inoculating the cells, draw two 2-ml samples from the bioreactor culture. Transfer 1 ml of each sample into a 1.5-ml cuvette.
? TROUBLESHOOTING

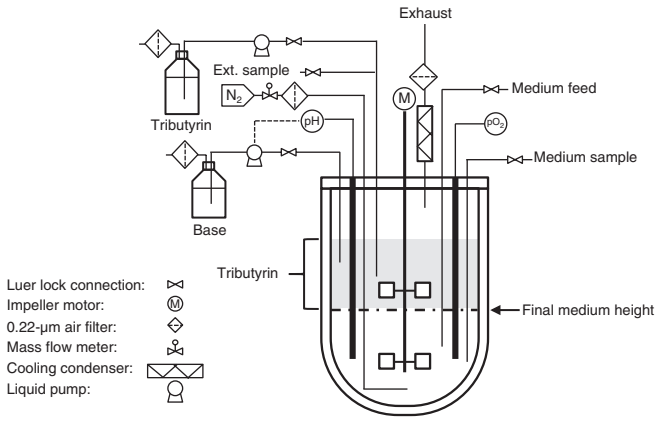


Figure 3 | Bioreactor setup for extractive fermentation without continuous concentrated media feeding.

4| In absorbance mode, blank a cuvette spectrophotometer with deionized water at the 600-nm wavelength.

5| Measure the sample absorbance (OD) at 600 nm, and dilute it if necessary with deionized water. Representative OD_{600} data for the fermentation is shown in **Figure 4**.

6| Filter the remaining sample volume with a 1-ml syringe through a 0.22-µm syringe filter into an HPLC vial and cap. High cell densities may make filtration difficult.
▲ CRITICAL STEP Centrifuging the sample for 3 min at 4,600–15,800*g* at 25 °C will pellet the cells, making filtration of the supernatant easier.

7| Analyze the fermentation broth HPLC sample as previously described in the HPLC analysis section of Equipment Setup.

8| Allow the culture pH to decrease from 5.8 to 5.0 over the first 4–6 h of fermentation, as measured by the online bioreactor pH probe. Then, adjust the pH of the bioreactor to ≥5.0 using 5 M KOH (20–60 ml of KOH will be consumed over the course of the fermentation).

9| Repeat sampling periodically throughout the fermentation (every 4–8 h).

Addition of extractant ● TIMING 30 min
▲ CRITICAL The extractant is added after the cells begin to produce solvents. This typically takes ~15–20 h, and it can be observed by an increase in the culture pH >5.05.
10| Sparge N_2 gas at 1 liter per hour through 1 liter of tributyrin for 1 h before addition to the bioreactor to ensure that the extractant is anaerobic.

11| Decrease the bioreactor agitation to 50 r.p.m. and maintain it for the remainder of the fermentation.

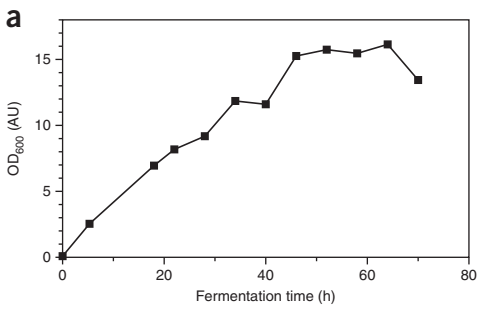
12| Attach one end of the 4.8-mm (i.d.) silicone tube line to the bioreactor using a Luer lock connection. Make the connection aseptic by coating in 70 wt% ethanol.

13| Feed the tubing through a peristaltic pump, and immerse the other end into the anaerobic tributyrin.

14| Quickly pump (>50 ml min^{-1}) the entire 1 liter of anaerobic tributyrin into the bioreactor.

15| Disconnect the extractant line from the bioreactor, cap the Luer lock connection, coat the connection with 70 wt% ethanol and turn off N_2 sparging in the bioreactor.

Extractant sampling ● TIMING 30 min, repeated every 8 h for 40 h
16| Draw three 1.5-ml samples from the extractant phase of the bioreactor through the extractant sampling line.



b

Time (h)	OD_{600} measured (AU)	Dilution factor	Actual OD_{600} (AU)
0	0.087	1	0.087
5.3	0.508	5	2.54
18	0.463333333	15	6.95
22	0.409	20	8.18
28	0.459	20	9.18
34	0.474	25	11.85
40	0.386666667	30	11.6
46	0.508666667	30	15.26
52	0.524666667	30	15.74
58	0.515333333	30	15.46
64	0.538	30	16.14
70	0.448	30	13.44

Figure 4 | Monitoring fermentation growth and culture stability with absorbance at 600 nm. (**a**) Representative OD_{600} values versus fermentation time for the *C. acetobutylicum* extractive fermentation. (**b**) Representative dilution factors and measured OD_{600} values. AU, absorbance units.

17| Prepare aliquots of the samples in 1.7-ml microcentrifuge tubes.

18| Spin the tubes at 9,400*g* for 3 min to phase-separate the cells and water from the extractant sample.

19| Collect and filter only the extractant phase of the spun-down sample with a 1-ml syringe through a 0.22-µm filter into a GC vial and cap.

20| Analyze the extractant phase sample as previously described in the GC analysis section of Equipment Setup.

21| Repeat sampling periodically throughout the fermentation (every 8 h).
▲ **CRITICAL STEP** Samples should be analyzed immediately on GC or stored at –20 °C to prevent loss of volatiles.

Bioreactor feeding ● TIMING 30 min (plus 40 h of incubation time)
▲ **CRITICAL** 24 h after inoculation, the bioreactor's glucose concentration should be monitored and adjusted every 4–8 h.
22| Use a 5-ml syringe (sterile) to remove 1 ml of culture; place it in a microcentrifuge tube.

23| Centrifuge the sample at 4,600–9,400*g* for 3 min at 25 °C to pellet the cells.

24| Dilute 100 µl of the sample supernatant into 900 µl of deionized water.

25| Measure glucose concentration on a YSI Biochemistry Analyzer.

26| If the glucose concentration is <25 grams per liter, add enough concentrated *Clostridium* medium to raise the concentration >35 grams per liter.
▲ **CRITICAL STEP** If accurate continuous feeding is possible with your bioreactor system, flow concentrated medium at an initial rate of 6 ml h^{-1} once the glucose concentration falls below 40 grams per liter. Every 6–8 h, measure the glucose concentration and adjust the flow rate of the medium to maintain glucose concentrations between 15 and 30 grams per liter.
? TROUBLESHOOTING

27| Continue monitoring and feeding the reactor until the cells stop consuming glucose or the OD_{600} of the fermentation broth drops markedly (2–4 units in 4–6 h).

Collection of extractant for distillation ● TIMING 30 min
▲ **CRITICAL** Once the fermentation is complete, the extractant phase is collected for distillation. It is very important that the collected extractant contain few to no cells or little to no excess water.
28| 1 h Before collecting the extractant, turn off agitation in the bioreactor to ensure a clean extractant:broth interface.

29| Immerse one end of a 4.8-mm silicone tubing line into the extractant phase. Make sure that the line remains 1-inch above the extractant:broth interface.

30| Feed the tubing through a peristaltic pump, and place the other end into a 1-liter collection bottle.

31| Pump out the tributyrin, making sure not to collect any fermentation broth or cells.

32| Distill the extractant shortly after collection or store it at –20 °C.

ABE distillation from the extractant phase ● TIMING 2 h (plus 4 h of incubation time)

33| Place 100 ml of extractant phase from the ABE fermentation in a 250-ml round-bottom flask. Assemble the distillation apparatus as shown in **Figure 5**. Note that hose clamps should be used to ensure fixation of the water lines to the distillation condenser.

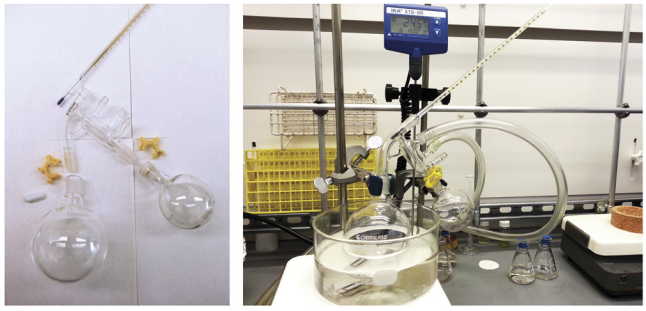


Figure 5 | Distillation apparatus components and assembled setup.

34| With the round-bottom flask immersed in a silicone oil heating bath, slowly increase the temperature to 150 °C and collect the distillate in the receiver flask. This distillate will contain the acetone (boiling point (bp) = 56 °C), ethanol (bp = 78 °C), water (bp = 100 °C) and butanol (bp = 118 °C).

35| Repeat Steps 31 and 32 with the remaining extractant phase.

36| Place all of the distillate into a 50-ml round-bottom flask and attach it to the distillation apparatus.

37| Begin slowly heating the distillation apparatus using the silicone heating bath to 80 °C by collecting the distillate in a clean receiver flask to separate the acetone and ethanol from the water and butanol.
▲ **CRITICAL STEP** Do not allow the distillation temperature to rise above 80 °C.

38| After the liquid stops accumulating in the receiver flask, remove the flask, seal it and store it at –20 °C. Install a new clean receiver flask to capture the butanol.

39| With the new receiver flask installed, begin raising the distillation apparatus temperature to 150 °C. Hold the apparatus temperature at 150 °C until liquid stops accumulating in the receiver flask, which takes at least 30 min. The distillate in the receiver flask will form two immiscible phases.

40| Remove the receiver flask and carefully decant the butanol-rich upper phase (density = 0.81) into the previously collected 80 °C distillate solution.

41| Dry this mixture over 2 g of activated 3 Å molecular sieves for 1 h.

42| Remove the liquid phase and measure the water content using Karl Fisher titration[18].
▲ **CRITICAL STEP** The water content must be <0.5 wt% before running the alkylation reaction. Repeat drying over 3 Å molecular sieves if necessary.

43| If you are not performing the alkylation reaction immediately, store the solvent mixture in a sealed flask at –20 °C; solvents will slowly evaporate if they are left at room temperature. Use them within ~1 month after refrigeration.

Catalyst preparation ● TIMING 20 min (plus 13 h of incubation time)

44| Calcine 8 g of hydrotalcite in a muffle furnace at 550 °C for 3 h using a 2 °C min^{-1} heating ramp, and then allow it to cool to room temperature.

45| Weigh 0.40 g of palladium(II) nitrate dihydrate (1.50 mmol) into a beaker and dissolve it into 4 ml of deionized water.

46| Weigh 8 g of calcined hydrotalcite into a mortar.

47| By means of a syringe (or addition funnel), add the palladium solution to the calcined hydrotacite in 0.2-ml aliquots while grinding with a pestle, until all of the solution has been absorbed. Add an additional 10 ml of water in 0.2-ml aliquots while grinding, so that a thin paste is obtained.
? TROUBLESHOOTING

48| Place the mortar in a drying oven at 100 °C for 3 h.

49| After drying, finely grind the obtained solid using a pestle and transfer it to a muffle furnace for calcination.

50| Calcine the catalyst under air at 550 °C for 5 h using a 2 °C min^{-1} ramp, followed by cooling to room temperature.

51| Transfer the solid to a calcination boat, and place it into a tubular furnace for reduction. Heat the sample to 550 °C for 2 h using a 2 °C min^{-1} ramp under hydrogen (flow rate: 100 ml min^{-1}), followed by cooling to room temperature under an inert gas (helium flow rate: 100 ml min^{-1}).

! CAUTION When cooling, turn on helium before turning off hydrogen flow; otherwise, fire can occur.

? TROUBLESHOOTING

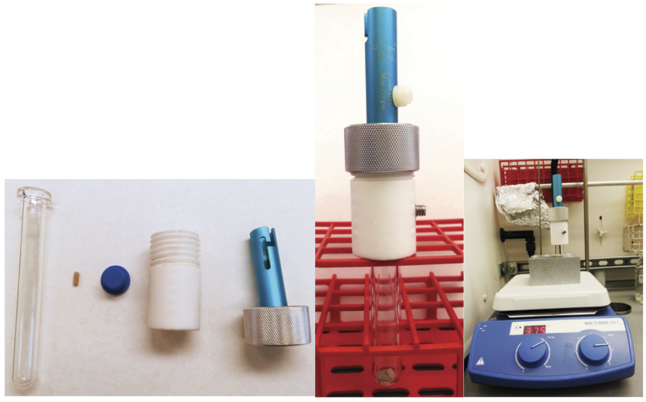


Figure 6 | Assembly of Q-tube reactor and parallel synthesizer.

■ **PAUSE POINT** After reduction of the catalyst, store it in an airtight sample vial. The catalyst will require recalcination and reduction after 1 month's storage.

Catalytic ABE reaction setup ● TIMING 30 min (plus 20 h of incubation time)

▲ **CRITICAL** This reaction can be performed either with 50 mg of distilled fermentation products from Step 39 in toluene (1.5 ml) or with the synthetic mixture outlined in Steps 51–53.

52| Place a dry Q-tube, equipped with a stir bar, in a test tube stand (**Fig. 6**).

53| Weigh 0.35 g of 2 wt% Pd-HT and place it in the Q-tube.

54| Weigh 0.092 g of ethanol (2 mmol) and place it in the Q-tube.

55| Weigh 0.267 g of acetone (4.6 mmol) and place it in the Q-tube.

56| Add 0.548 g of 1-butanol (7.4 mmol) to the Q-tube.

57| Add 0.1 g of internal standard (dodecane) to the Q-tube.

58| Seal the Q-tube with the butyl rubber septum followed by the Q-tube sleeve fitted with the metal pressure adapter 1240.

59| Place the Q-tube in a preheated parallel synthesizer at 240 °C; stir it vigorously for 20 h.

? TROUBLESHOOTING

60| Remove the Q-tube from the parallel synthesizer and allow it to cool for 30 min.

? TROUBLESHOOTING

61| By using a catch bottle fitted with a needle, release the pressure in the Q-tube by piercing the butyl rubber septum.

! CAUTION Q-tubes can build substantial pressure during the reaction and can rupture. In addition to standard personal protective equipment, wear thick leather gloves and use a blast shield while piercing septa.

62| Dilute the Q-tube with tetrahydrofuran and mix the reaction mixture by means of a spatula.

63| Centrifuge the reaction mixture for 3 min at 25 °C to force the catalyst to the bottom.

64| Transfer the clear supernatant to a GC vial and dilute it with tetrahydrofuran.

Determination of yield

65| Determine the experimental weight of the ketones by injecting an aliquot of the material obtained in Step 60 (100 µl in 1 ml of tetrahydrofuran) into the gas chromatograph equipped with a FactorFour column VF-5ms after calibrating the retention time of the ketones with the authentic sample. Monitor the peaks with an FID detector.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
3	Cells do not grow in the bioreactor	The conditions are not anaerobic	Verify that only N_2 is being sparged into the vessel and check for leaks in the N_2 line
	Culture pH continues to decrease below 5.0	Base pump failure or pump control sensitivity	Look for cracks in the base line and replace the tubing Adjust the integral parameter of pump control
26	Extractant layer is full of culture broth	Gas sparging is still on	Turn gas sparging off and reduce agitation rate
47	Hydrotalcite forms large clumps while metal solution is added	Solution is being added too quickly	Add smaller aliquots of liquid and grind more thoroughly after each addition
51	Water forms in the glass tube during reduction	A small amount of spilt catalyst is coating the downstream end of the reduction tube	Thoroughly clean the glass tube between uses, and always insert boats from the upstream end of the tube
59	The reaction mixture is not stirring in Q-tube	The catalyst has formed unsuspended clumps and the stir bar is stuck	Gently tap the tube against a hard surface
60	The Q-tube is stuck in parallel synthesizer block	The glass has expanded under heating	Blow lab air on the Q-tube while pulling gently to remove it

● TIMING

Reagent preparation: 2 h (plus 8 h of incubation time)
Bioreactor assembly: 2 h (plus 6 h of incubation time)
Steps 1–9, batch-phase preparation: 30 min (plus 18 h of incubation time)
Steps 10–15, addition of extractant: 30 min
Steps 16–21, extractant phase sampling: 30 min (repeated every 8 h for 40 h)
Steps 22–27, bioreactor feeding: 30 min (repeated every 4–8 h for 40 h)
Steps 28–32, collection of the extractant phase: 30 min
Steps 33–43, ABE distillation from the extractant phase: 2 h (plus 4 h of incubation time)
Steps 44–51, catalyst preparation: 20 min (plus 13 h of incubation time)
Steps 52–65, catalytic ABE reaction setup: 30 min (plus 20 h of incubation time)
Box 1, Bacteria inoculation and preculture: 1 h (plus 24 h of incubation time)
Box 2, Fermentor setup: 2 h (plus 6 h of incubation and sparging time)

ANTICIPATED RESULTS

When *C. acetobutylicum* (ATCC 824) was grown out (**Fig. 4**) and fermented with glyceryl tributyrate at a 1:1 volume

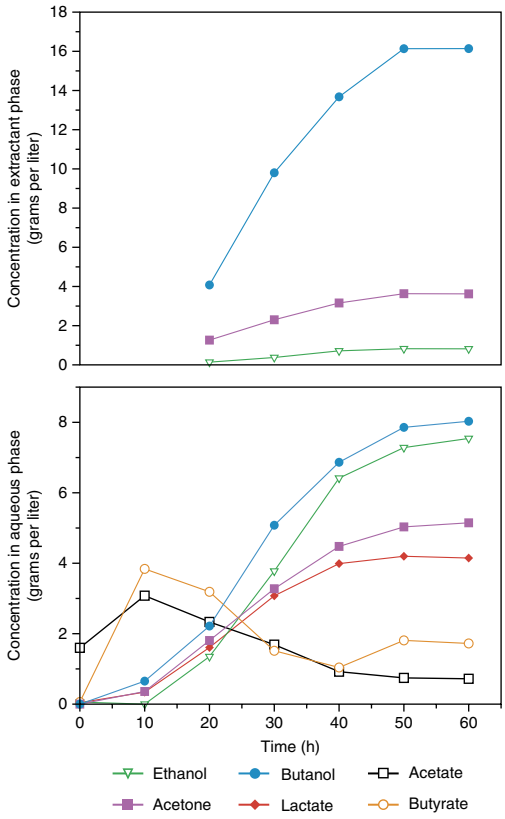


Figure 7 | Major metabolite concentrations in both phases of a *C. acetobutylicum* extractive fermentation. The major carbon source for the fermentation was glucose. Concentrations calculated by GC and HPLC methods described in this protocol.

TABLE 2 | ABE reaction yield.

Ketone products	Weight of ketones (g)	Molar yield with respect to acetone (%)
2-Pentanone	0.010	2.6
2-Heptanone	0.049	9.4
4-Heptanone	0.055	10.4
4-Nonanone	0.075	11.4
6-Undecanone	0.185	23.6
2-Pentanol	0.012	3.0
2-Heptanol	0.027	5.1
4-Heptanol	0.072	13.5
6-Undecanol	0.052	6.6

ratio, as described in the above protocol, 24.8 g of butanol, 8.9 g of acetone and 7.3 g of ethanol were produced. Of these, 16.4 g of butanol, 3.7 g of acetone and 0.8 g of ethanol partitioned into the glyceryl tributyrate phase. Production of 41.0 g of solvents required the consumption of 105 g of glucose. Solvent production ceased 60 h after inoculation of the culture into the bioreactor. **Figure 7** shows a time course of major metabolite concentrations in both phases of the fermentation.

When the ABE alkylation reaction is carried out with acetone (4.6 mmol, 0.267 g), 1-butanol (7.4 mmol, 0.548 g) and ethanol (2 mmol, 0.92 g) using Pd-HT (0.350 g) in a Q-tube, as described in the above protocol, a mixture of hydrocarbon ketone in the following composition is obtained (**Table 2**; the total yield is 86%).

Similar results can be obtained when the distilled fermentation mixture is used as long as the initial water content is <1.0 wt%, as determined by Karl Fisher titration.

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