A Sustainable Chemicals Manufacturing Paradigm Using CO₂ and Renewable H₂

Rajesh Reddy Bommareddy, Yanming Wang, Nicole Pearcy, Martin Hayes, Edward Lester, Nigel P. Minton, Alex V. Conradie

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HIGHLIGHTS
Stable and continuous gas fermentation using C. necator
Carbon-efficient biocatalysis from CO₂ and H₂ to commodity chemicals
Heat integration overcomes energy inefficiency of biological CO₂ fixation
A Sustainable Chemicals Manufacturing Paradigm Using CO₂ and Renewable H₂

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SUMMARY
The chemical industry must decarbonize to align with UN Sustainable Development Goals. A shift toward circular economies makes CO₂ an attractive feedstock for producing chemicals, provided renewable H₂ is available through technologies such as supercritical water (scH₂O) gasification. Furthermore, high carbon and energy efficiency is paramount to favorable techno-economics, which poses a challenge to chemo-catalysis. This study demonstrates continuous gas fermentation of CO₂ and H₂ by the cell factory, Cupriavidus necator, to (R,R)-2,3-butanediol and isopropanol as case studies. Although a high carbon efficiency of 0.75 [(C-mol product)/(C-mol CO₂)] is exemplified, the poor energy efficiency of biological CO₂ fixation requires ~8 [(mol H₂)/(mol CO₂)], which is technoeconomically infeasible for producing commodity chemicals. Heat integration between exothermic gas fermentation and endothermic scH₂O gasification overcomes this energy inefficiency. This study unlocks the promise of sustainable manufacturing using renewable feedstocks by combining the carbon efficiency of bio-catalysis with energy efficiency enforced through process engineering.

INTRODUCTION
The chemical industry has served economic growth well over many decades, much of the chemical industry has become misaligned with the United Nation’s (UN) Sustainable Development Goals, notably Sustainable Industrialization and Climate Action (Axon and James, 2018). Current large-scale manufacturing processes suffer from a reliance on finite fossil reserves, high energy consumption, and poor overall catalytic selectivity. Circular economies are markedly absent and net greenhouse gas emissions exacerbate climate change (Keijer et al., 2019).

Given the chemical industry needs to decarbonize, the use of CO₂ as a carbon feedstock for producing chemicals has significant synergy with UN Sustainable Development Goals, provided a renewable supply of reducing power is available from either H₂ or H₂O. Renewable H₂ can be produced via a network of sustainable technologies, including (1) biomass pyrolysis-gasification (Dou et al., 2019), (2) dark fermentation of complex carbohydrates (Boboescu et al., 2016), (3) supercritical water gasification (Oklie et al., 2019), and (4) megawatt-scale water electrolysis (Schmidt et al., 2017). Such renewable H₂ can be used to produce methanol from CO₂ chemo-catalytically, where a typical reactor has a methanol outlet composition of ~61% by mole of the total carbon products (Toyir et al., 2009). This renewable methanol can be converted to C₂–C₄ olefin chemical building blocks using SAPO-34 zeolite catalysts, noting a typical reactor has a propene outlet composition of 39% by weight of the total carbon products in commercial practice. In addition to low selectivity, the Methanol to Olefins (MTO) process suffers from rapid catalyst coking, necessitating continuous regeneration of the catalyst within the fluidized bed reactor (Tian et al., 2015). The MTO process’ greater selectivity for propene creates opportunities to produce C₃ and C₄ alcohol solvents. Isopropanol can be produced via the hydration of the C₃ propene fraction, whereas C₄ alcohols can be produced through hydroformylation of propene using the OxoSM Process with ~85% selectivity for the linear C₄ product over the branched C₄ by-product (Tudor and Shah, 2017). In addition to the techno-economic challenges posed by low overall selectivity and catalyst deactivation, the high temperature and pressure processing associated with the chemo-catalytic conversion of CO₂ and H₂ to C₃ and C₄ products is energy intensive (Toyir et al., 2009; Tian et al., 2015; Tudor and Shah, 2017).

The chemical industry must decarbonize to align with UN Sustainable Development Goals. A shift toward circular economies makes CO₂ an attractive feedstock for producing chemicals, provided renewable H₂ is available through technologies such as supercritical water (scH₂O) gasification. Furthermore, high carbon and energy efficiency is paramount to favorable techno-economics, which poses a challenge to chemo-catalysis. This study demonstrates continuous gas fermentation of CO₂ and H₂ by the cell factory, Cupriavidus necator, to (R,R)-2,3-butanediol and isopropanol as case studies. Although a high carbon efficiency of 0.75 [(C-mol product)/(C-mol CO₂)] is exemplified, the poor energy efficiency of biological CO₂ fixation requires ~8 [(mol H₂)/(mol CO₂)], which is technoeconomically infeasible for producing commodity chemicals. Heat integration between exothermic gas fermentation and endothermic scH₂O gasification overcomes this energy inefficiency. This study unlocks the promise of sustainable manufacturing using renewable feedstocks by combining the carbon efficiency of bio-catalysis with energy efficiency enforced through process engineering.

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Microbial cell factories produce biocatalysts (enzymes) and use the cell’s energy carriers to synthesize products via these non-native biochemical pathways. Such bio-catalysis presents opportunities to reinvent chemicals manufacturing using sustainable feedstocks and renewable energy, harnessing the high catalytic selectively of microbial cell factories at low temperature and pressure (Hedstrom, 2010). Although several acetogenic cell factories are able to fix CO₂ using H₂ anaerobically, these cell factory platforms suffer from energetic limitations (Molitor et al., 2017) and the production of fermentative by-products such as acetate (Hoffmeister et al., 2016). Conversely, Cupriavidus necator (formerly, Alcaligenes eutrophus and Ralstonia eutropha) is a chemolithoautotrophic bacterium capable of aerobic, autotrophic growth using CO₂ as the sole carbon source, H₂ as electron donor, and O₂ as the electron acceptor (Brigham, 2019). When the genes producing C. necator’s natural carbon sink, polyhydroxybutyrate (phb), are attenuated, the cell accumulates pyruvate as the central metabolite under nutrient limitation, which can be redirected to a number of different carbon products (Steinbüchel and Schlegel, 1989). Consequently, metabolic engineering of C. necator to produce chemicals from CO₂ and H₂ has demonstrated promise, most notably in the production of (1) 2-hydroxyisobutyrate for Plexiglas (Przybylski et al., 2012), (2) isobutanol (Brigham et al., 2013), (3) 3-methyl-1-butanol (Li et al., 2012), (4) methyl ketones (Müller et al., 2013), (5) isopropanol (Marc et al., 2017), (6) α-humulene (Krieg et al., 2018), and (7) acetoin (Windhorst and Gescher, 2019). These studies have contributed appreciably to advancing the metabolic engineering of C. necator as a platform for using CO₂ as a carbon feedstock, noting that the focus was not on process engineering considerations. As such, these studies were demonstrated at low cell density in batch operation, which is not aligned technoeconomically with continuous manufacturing. Additionally, these studies have not addressed the energy inefficiency of biological CO₂ fixation, where the high H₂ utilization makes the process technoeconomically infeasible for producing commodity chemicals (Emerson and Stephanopoulos, 2019).

The objective of this study has been to demonstrate integrated, continuous production of chemicals from CO₂ using C. necator as the microbial cell factory. Aligning with the continuous operating paradigm of the chemical industry, this paper is the first to demonstrate the stable and continuous bio-manufacture of chemicals from CO₂ using C. necator as carbon-efficient cell factory. Furthermore, this study is the first to demonstrate the use of process engineering to overcome the techno-economic hurdle associated with the energy inefficiency of biological CO₂ fixation. The following sections outline the metabolic engineering, continuous gas fermentation, and rigorous process simulation, which exemplify carbon and energy-efficient continuous bio-manufacture through two case studies producing (R,R)-2,3-butanediol (BDO) and isopropanol (IPA).

**RESULTS**

**Metabolic Engineering**

Microbial cell factories are able to produce a large number of products via their biochemical networks from a variety of carbon feedstocks. BDO and IPA were selected as the two case studies for continuous autotrophic fermentation in this work, given both biochemical pathways have been extensively characterized (Ji et al., 2011; Marc et al., 2017). The biochemical pathways that convert the central metabolite pyruvate to either BDO or IPA are detailed in Figure 1A. The biochemical pathways depicted in Figure 1B were overexpressed as a single operon from either a plasmid or chromosomal integration, employing the synthetic biology methods described in the Transparent Methods section.

C. necator’s natural carbon sink pathway to polyhydroxybutyrate (phb) was knocked out by deleting the operon *phaC1B1* for the BDO cell factory and the genes *phaC1B1* for the IPA cell factory (Peplinski et al., 2010; Raberg et al., 2014; Müller et al., 2013), thereby redirecting carbon flux and reducing equivalents to the fermentation product. Also, given the reported degradation of BDO’s precursor acetoin by C. necator via what would constitute a competing pathway (Freund et al., 1989), the acoXABC gene cluster was deleted in the BDO cell factory.

The performance evaluations of these BDO and IPA cell factories in heterotrophic shake flask culture are summarized in Table 1 and Figure 2, demonstrating the carbon split using fructose as carbon source. Given that chromosomal integration is associated with greater genetic stability in C. necator (Voss and Steinbüchel, 2006; Gruber et al., 2014), the performance of the expressed operons (Figure 1B) was assessed for both plasmid-based and chromosomally integrated cell factories. The cumulative specific fructose uptake rate was comparable across plasmid, integrated and control strains, where the biomass synthesis was controlled via nitrogen limitation. In shake flasks, the integrated BDO cell factory had similar yield and
productivity performance to the plasmid-based cell factory, whereas the IPA plasmid-based cell factory outperformed the integrated cell factory. The greater genetic stability of integrated cell factories is best suited to continuous fermentation, and a single copy integration also provided for a better comparison of performance between BDO and IPA. Therefore, both the BDO and IPA integrated cell factories were taken forward into continuous fermentation using CO₂ and H₂ as per the Transparent Methods section.

**Continuous Gas Fermentation**

Microorganisms often show reduced tolerance to the accumulation of solvent products such as BDO or IPA. Increasing IPA concentrations in fermentation are detrimental to *C. necator*’s growth above 15 g/L (Marc et al., 2017), recognizing that stripping of IPA into the bioreactor’s off-gas increases as the aqueous concentration increases. Also, in continuous fermentation, the IPA is further diluted from the bioreactor. The low volatility of BDO makes its accumulation in the bioreactor’s aqueous phase a greater concern. Consequently, shake flask experiments revealed that, above a BDO concentration of 30 g/L, the growth rate of *C. necator* H16 is impaired (Figure S1). Therefore, without resorting to genetic modification, the BDO concentration in continuous bioreactors needs to be controlled through dilution alone.

The continuous, autotrophic fermentation results were generated using the bioreactor experimental setup shown in Figure 3, outlining the decoupled, multi-loop SISO (single-input, single-output) process control strategy for intensifying the process within the flammability safety constraints. The specific CO₂ uptake...
rates and specific productivities on a biomass (DCW) basis are trended in Figure 4, thereby allowing for
comparison between BDO and IPA synthesis against the ΔphaC1AB1ΔacoXABC control strain. For the
BDO cell factory, meso-2,3-butanediol is the principal by-product, indicative of promiscuous secondary
alcohol dehydrogenase activity accepting acetoin as substrate. Acetone is the principal by-product for
the IPA cell factory, indicating that the secondary alcohol dehydrogenase is the rate-limiting step in the
assembled pathway. The control strain produces no products or by-products. The molar ratios of \( \frac{H_2}{CO_2} \) and \( \frac{O_2}{CO_2} \) reflect the specific requirement for \( H_2 \) as electron donor and \( O_2 \) as electron
acceptor (Figure 5). The steady-state \( CO_2 \) uptake rate for both case studies demonstrated a specific uptake
rate of 3–4 \( \text{[(mmol C)/(g DCW h)]} \), consuming \( \approx 8 \text{[(mol H}_2\text{)/(mol CO}_2\text{)]} \) and \( 2–3 \text{[(mol O}_2\text{)/(mol CO}_2\text{)]} \). An
\( \approx 8 \text{[(mol H}_2\text{)/(mol CO}_2\text{)]} \) molar ratio reflects the reducing power required to fix \( CO_2 \) and produce the product,
generating significant heat owed to the exothermic reaction. Alongside this measure of energy efficiency,
the carbon split to product, by-product, and biomass summarizes the carbon efficiency (Table 2 and Fig-
ure 6). As such, these data are the first to demonstrate genetically stable and continuous production in
C. necator via chromosomal integration of non-native genes. The calculation methodology for gas uptake
rates, steady-state dilution rate, and carbon fluxes for the continuous, autotrophic fermentations is
described in Figure S2. Given the results in Figures 4 and 5 represent calculated data incorporating several
sensors, analyses, and calibration standards, two thousand Monte Carlo simulations were undertaken to
determine the 90% confidence limits denoted by error bars in Figures 4 and 5. The histogram outputs
from the Monte Carlo simulations are contained in Figure S3.

### Process Simulation and Systems Biology

**Heat Integration of Gas Fermentation with Supercritical Water Gasification**

Gas fermentation is a highly exothermic process owed to the cascade of electrons through the biochemical
network (Figure 1A) from the electron donor, \( H_2 \), to the final electron acceptor, \( O_2 \) (Tanaka et al., 1993). The
\( CO_2 \) is reduced to a number of carbon sinks, typically biomass, the fermentation product, and by-products. Super-
critical water (\( \text{scH}_2\text{O} \)) gasification is a hydrothermal technology converting renewable carbon feedstocks,
such as wet lignin biomass, to \( CO_2 \) and \( H_2 \) at supercritical pressure and temperature, i.e., 240 bar (a) and
375\(^\circ\)C (Rodriguez Correa and Kruse, 2018). The process is highly endothermic and the renewable \( H_2 \) produced
by the \( \text{scH}_2\text{O} \) reactor originates from both the hydrocarbon feedstock and the \( \text{scH}_2\text{O} \). A heat pump can be em-
ployed to facilitate the energy (heat) flow from a low temperature (gas fermentation) to a high temperature
(\( \text{scH}_2\text{O} \) gasification) via a thermal cycle. The integrated process was rigorously simulated in Aspen HYSYS, sum-
marized in Figure 7 and detailed in Figure 8 using the lignin model compound, guaiacol, as the waste carbon
feedstock. From the heat pump cycle depicted in Figure 7, a suitable heat carrying fluid (isopentane) is evapo-
rated at low pressure in an evaporator by the bioreactor’s heat of reaction (4,004 kW/ton guaiacol), resulting in a
substantial increase in the isopentane’s enthalpy (energy) at constant temperature. The isopentane vapor is com-
pressed to a higher pressure via a compressor, further increasing the isopentane’s enthalpy owed to the heat of
compression (175 kW/ton guaiacol). Further energy is transferred to the vapor via a series of heat exchangers.
The \( \text{scH}_2\text{O} \) Recovery Heat Exchanger recovers heat from the \( \text{scH}_2\text{O} \) reactor’s effluent (5,565 kW/ton guaiacol),
and the Heat Pump Recovery Heat Exchanger recovers heat from the isopentane returning after heating the
\( \text{scH}_2\text{O} \) reactor feed to supercritical temperature. Thereafter, the temperature of the isopentane is greatly
increased in a combustion chamber (3,952 kW/ton guaiacol), fired by a fraction of the renewable \( H_2 \) generated

| Performance Parameter | Unit | 2,3-Butanediol | Isopropanol |
|-----------------------|------|--------------|-------------|
|                       |      | Plasmid      | Integrated  | Host         | Plasmid | Integrated | Host |
| Cumulative specific fructose uptake rate | [mmol C]/(g DCW h)] | 9.6 ± 1.3 | 9.7 ± 1.2 | 8.8 ± 1.1 | 10.2 ± 1.8 | 9.9 ± 1.6 | 9.1 ± 1.1 |
| Cumulative molar carbon efficiency (yield) | [C-mol product]/(C-mol fructose)] | Figure 2 |
| Final product titer in liquid phase | [g/L] | 1.9 ± 0.1 | 1.8 ± 0.1 | nd | 2.7 ± 0.2 | 1.8 ± 0.2 | nd |

Table 1. Microbial Cell Factory Performance in Heterotrophic Shake Flasks for (R,R)-2,3-Butanediol and Isopropanol Synthesis (Triplicate Biological Replicates)

nd designates not detected.
in the scH2O reactor. The high temperature of the isopentane allows heat transfer to the subcritical aqueous feed (3,660 kW/ton guaiacol), raising the sub-critical feed to the scH2O reactor to supercritical conditions. After the Heat Pump Recovery Heat Exchanger, the vapor is condensed in a condenser (scH2O Reactor Pre-heater), which pre-heats the feed to the scH2O reactor (10,036 kW/ton guaiacol). Finally, the liquid isopentane is expanded to lower pressure over a valve for evaporation in the Heat Pump Evaporator by the bioreactor’s heat of reaction. The thermal cycle linking the low-temperature energy source, i.e., the exothermic gas fermentation, and the high-temperature energy sink, i.e., the endothermic scH2O gasification, is subsequently repeated.

The process integration into the heat pump cycle of (1) the combustion chamber, (2) air compression via a turbo-expander, and (3) renewable energy generation via a turbine is detailed in Figure 8. The integrated process was rigorously simulated in Aspen HYSYS, feeding the waste carbon from the High-Pressure Pump (339 kW/ton guaiacol) to the scH2O Reactor, where CO2 and H2 are generated from the carbon feedstock modeled as the lignin model compound, guaiacol. The high-pressure CO2 & H2 stream is expanded over the turbo-expander, using the generated 575 kW/ton guaiacol to compress air as the oxygen source for gas fermentation and the combustion chamber. The thermal cycle linking the low-temperature energy source, i.e., the exothermic gas fermentation, and the high-temperature energy sink, i.e., the endothermic scH2O gasification, is subsequently repeated.

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**Systems Biology for BDO Synthesis Using CO2 & H2 and Guaiacol as Sole Energy and Carbon Sources**

Rather than gasifying lignin, a next best alternative technology within the context of more conventional heterotrophic fermentation would be a process that converts lignin to guaiacol, thereafter converting the guaiacol to BDO via the 3-oxoadipate pathway. Shen et al. (2020) demonstrated that lignin can be selectively converted to guaiacol as an alternate technology to gasification. C. necator is capable of degrading...
lignin monomers such as catechol (Wang et al., 2014). However, a microbial cell factory using the lignin model compound guaiacol as the sole energy and carbon source has not been reported. Mallinson et al. (2018) uncovered the O-demethylase reaction that allows for guaiacol catabolism in bacteria, characterizing kinetics that suggests guaiacol catabolism is constrained by the conversion of guaiacol to catechol. The required biochemical network for the synthesis of BDO from guaiacol in C. necator H16 is shown in Figure S4. Guaiacol is catabolized via the 3-oxoadipate pathway to succinyl-CoA and acetyl-CoA, after which the carbon flux is directed to the cell’s TCA cycle. Pyruvate is produced from malate via the malic enzyme as the metabolite precursor to BDO synthesis. Using a genome scale model for C. necator (Unpublished Data), which advances on the existing model proposed by Park et al. (2011), comparative Flux Balance Analysis (FBA) simulations were run using (1) guaiacol as the sole carbon and energy source and (2) CO₂ and H₂. For CO₂ and H₂, the FBA simulation accurately predicted the O₂ uptake rate, H₂ uptake rate, and the BDO productivity as detailed in Table 2, providing confidence in the predictive power of the genome scale model. The FBA simulation for guaiacol predicted a molar carbon yield of 0.12 [(C mol product)/(C mol guaiacol)] (Table S6). Accordingly, the upstream processing for the guaiacol case was scaled to a guaiacol feed basis of 1,000 kg/h and simulated in Aspen HYSYS as shown for the conventional heterotrophic process flow sheet in Figure 9. Similarly, the upstream processing for the CO₂ & H₂ case was scaled to a guaiacol feed basis of 1,000 kg/h and simulated in Aspen HYSYS as shown for the autotrophic process flow sheet in Figure 8. The bioreactor scale-up for the guaiacol and CO₂ & H₂ Aspen HYSYS simulations is summarized in Table S7.

Figure 3. Continuous, Autotrophic Bioreactor Experimental Setup
Continuous bioreactor experimental setup, outlining the decoupled, multi-loop SISO (single-input, single-output) process control strategy for intensifying the continuous autotrophic bioreactor within the flammability safety constraints. The steady-state dissolved oxygen (AIC101) is controlled via the phosphate addition rate, maximizing the process intensification under phosphate limitation. Avoiding a flammable atmosphere in the headspace of the reactor, AIC102 controls the O₂ concentration in the headspace using the air flow.
DISCUSSION

High carbon and energy efficiency is essential to achieving favorable techno-economics when converting CO₂ to chemicals, which poses a significant challenge to conventional chemo-catalysis. Principally, the low overall selectivity of the Methanol to Olefin (MTO) process would hamper the sustainable production of C₃ and C₄ alcohols from CO₂. For the integrated cell factories producing BDO and IPA (Table 1 and Figure 2), the batch experiments using fructose as carbon source showed low molar carbon yields of 0.16–0.18 [(C mol product)/(C mol fructose)]. From Figure 2, the carbon sunk into biomass and by-products was low and a large fraction of carbon was released as CO₂, indicative of the reducing power required for the synthesis of BDO and IPA. Although fructose makes no net contribution to CO₂ emissions, a biogenic carbon source such as lignin would be more cost-effective. However, for the lignin model compound, guaiacol, systems biology simulation for heterotrophic catabolism predicts a similar molar carbon yield of 0.12 [(C mol product)/(C mol guaiacol)]. Scaling this conventional heterotrophic process as in Figure 9 produces 158 kg of BDO per ton of guaiacol, requiring 744 kW of electricity for air compression and 1,638 kW of electricity for the ammonia chiller per ton of guaiacol. The total cooling tower duty amounts to 7,702 kW, adding to the operating cost burden. Despite using renewable feedstocks, such a process could not be described as sustainable.

In contrast, the BDO and IPA cell factories in continuous gas fermentation have substantially improved carbon efficiency, given the reducing power from H₂ is fed separately from the oxidized CO₂ feed. The BDO

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**Figure 4. Specific CO₂ uptake rates and specific carbon productivities for continuous, autotrophic fermentations**

Specific CO₂ uptake rate (CUR) and specific carbon productivity on a biomass basis during the synthesis of (R,R)-2,3-butanediol and isopropanol in continuous autotrophic fermentation, compared with the ΔphaC1AB1 ΔacoXABC control. Specific productivities are comparable, noting the greater carbon overflow to by-product for the isopropanol cell factory. Error bars determined as per Figure S3.
and IPA cell factories achieved high molar carbon yields of 0.75 [(C mol product)/(C mol CO₂)] and 0.61 [(C mol product)/(C mol CO₂)], respectively (Table 2 and Figure 6), recognizing that the IPA cell factory would benefit from further metabolic engineering optimization. Approximately 0.15–0.2 [(C mol DCW)/(C mol CO₂)] is invested into the continuous production of bio-catalyst, negating the need to have a separate unit operation to regenerate catalyst as for the MTO process. Despite the stable and high overall carbon selectivity (Figures 4 and 6) aligned with the chemical industry’s production paradigm, the poor energy efficiency of biological CO₂ fixation requires considerable renewable H₂ at ~8 [(mol H₂)/(mol CO₂)] (Table 2 and Figure 5), which is techno-economically infeasible for producing commodity chemicals.

Several researchers have recognized that the energy inefficiency associated with biological carbon fixation is a hurdle to creating techno-economic processes based on gas fermentation (Bar-Even et al., 2012; Emerson and Stephanopoulos, 2019). The Calvin-Benson-Bassham (CBB) cycle is the dominant carbon fixation pathway given its prevalence within photosynthetic organisms. The CBB cycle’s dominance is owed to its advantaged kinetics over other carbon fixation pathways such as the reductive acetyl-CoA pathway prevalent in anaerobic acetogens. This kinetic advantage to reduce CO₂ into biomass and other metabolites comes at a substantial energy cost. For example, to produce 1 mole of acetate, 7.5 moles of H₂ is required by the CBB cycle as opposed to 4 moles of H₂ for the reductive acetyl-CoA pathway (Emerson and Stephanopoulos, 2019). Consequently, a number of augmented and artificial CO₂ fixation pathways have been proposed as a means of improving the energy

**Figure 5. Molar Uptake Ratios for Continuous, Autotrophic Fermentations**

Molar uptake ratios for H₂ (electron donor) and O₂ (electron acceptor) to CO₂ during the synthesis of (R,R)-2,3-butanediol and isopropanol in continuous autotrophic fermentation, compared with the ΔphaC1AB1 ΔacoXABC control. An ~8 [(mol H₂)/(mol CO₂)] molar ratio reflects the reducing power required to fix CO₂ and produce the product, generating significant heat owed to the exothermic reaction. Error bars determined as per Figure S3.
efficiency of biological C1 fixation cycles. Yu et al. (2018) proposed a malyl-CoA-glycerate (MCG) pathway to augment the CBB cycle, which reduces energy requirements by 22.5% to produce the central metabolite, acetyl-CoA. Implementing the MCG pathway would thus have potential benefit to this study’s IPA, but not the BDO, cell factory. More generally, Gleizer et al. (2019) were the first to introduce an entire CO2 fixation pathway into a heterotrophic cell factory by relying on CBB cycle enzymes and formate assimilation. This significant achievement in metabolic engineering imparted chemolithotrophic metabolism to E. coli. Given formate needs to be oxidized to CO2 at a molar ratio of \(-8\) [(mol formate)/(mol CO2 fixed)] to provide reducing power for the CBB cycle, the carbon efficiency of this formate-assisted pathway is low and the cell’s energy efficiency no better than the conventional CBB cycle. Furthermore, the production of formate from CO2 via electrolysis is challenged by appreciable electricity demand and by electrode poisoning of the noble metal catalyst (Lee et al., 2019), where megawatt-scale implementation would be as capital intensive as for H2O electrolysis (Schmidt et al., 2017). Despite their apparent thermodynamic promise, no artificial CO2 fixation pathways have been successfully implemented in a cell factory. The problem of poor energy efficiency needs to be solved another way.

Low-cost renewable H2 production is essential to achieving favorable techno-economics, although this is only part of the solution. Biomass pyrolysis-gasification needs to be implemented at considerable scale to justify the required solids handling capital investment, which may be mismatched with the more distributed

| Performance Parameter | Unit | BDO Microbial Cell Factory | IPA Microbial Cell Factory |
|------------------------|------|----------------------------|---------------------------|
| Specific CO2 uptake rate | [mmol CO2/gDCW-h] | 3.04 ± 0.12 | 3.99 ± 0.11 |
| Molar carbon efficiency (yield) | [(C-mol product)/(C-mol CO2)] | | Figure 6 |
| Carbon balance closure | [%] | 102.6 | 93.7 |
| H2/CO2 Molar ratio | [(mol H2)/(mol CO2)] | 7.91 ± 0.86 | 8.09 ± 0.74 |
| O2/CO2 Molar ratio | [(mol H2)/(mol CO2)] | 2.92 ± 0.1 | 1.85 ± 0.06 |
| Product in vapor phase | [-] mole fraction | 0 | 0.75 |
| Product in liquid phase | [g/L] | 32.0 ± 0.1 | 7.7 ± 0.2 |

Table 2. Microbial Cell Factory Performance in Continuous Autotrophic Fermentation for (R,R)-2,3-Butanediol and Isopropanol Synthesis

*BDO is 2,3-butanediol.
*IPA is isopropanol.

Figure 6. Molar Carbon Efficiencies for Continuous, Autotrophic Fermentations

Molar carbon efficiency (yield) of microbial cell factories in autotrophic fermentation, representing the carbon split using CO2 as carbon source in [(C-mol product)/(C-mol CO2)]. The by-product for the BDO strains is meso-2,3-butanediol and for the IPA strains is acetone. Table 2 summarizes additional performance parameters.
nature of smaller bio-manufacturing facilities (Dou et al., 2019). Dark H₂ fermentation of complex carbohydrates suffers from low process intensification (Boboescu et al., 2016), whereas megawatt-scale H₂O electrolysis is too capital intensive making large-scale H₂ production prohibitive (Schmidt et al., 2017). Supercritical H₂O gasification needs to overcome corrosion and fouling challenges but can be implemented cost-effectively at a smaller scale, demonstrates high reaction rates, and is not prohibitive from a capital investment perspective (Okolie et al., 2019). Cost-effective catalysis is key to economically viable H₂ production at temperatures ~400°C, where scH₂O presents opportunities to produce nano-catalyst in situ (Huang et al., 2019) and for the recycle of valuable metals from spent catalyst (Grumett, 2003). Pertinent to this study, in addition to serving as a source of renewable H₂, the highly endothermic scH₂O gasification reaction provides a heat sink for the highly exothermic gas fermentation.

The consumption of H₂ to fuel the CBB cycle’s kinetics generates a significant amount of heat at low temperature in bioreactors, which is conventionally removed via a chiller unit at the expense of electrical energy and high cooling water duty as shown in Figure 9. A heat pump can be employed to facilitate the energy (heat) flow from a low temperature (gas fermentation) to a high temperature (scH₂O gasification) via a thermal cycle as outlined in Figure 7 and detailed in Figure 8. Comparing the capital intensity of a conventional flowsheet (Figure 9) and the heat integrated flowsheet (Figure 8), Figure 8 has (1) a turbo-expander rather than a megawatt-scale air compressor, (2) a heat pump rather than a chiller thermal cycle, and (3) further energy recovery via a turbine. Reducing the operating cost burden associated with compression for gas fermentation, a turbo-expander supplies air for gas fermentation and the combustion chamber with no intrinsic electrical power consumption. In addition to producing 148 kg of BDO per ton of gasified guaiacol, comparable with guaiacol as sole carbon source (Figure 9), the process generates 566 kW of renewable electricity rather than consuming significant electrical power. From Figure 7, the overall heat duty of the scH₂O gasifier amounts to 13.7 MW/ton guaiacol. Without heat integration with gas fermentation, the combustion chamber would need to supply 58% of this heat duty, severely limiting the supply of H₂ per ton of guaiacol to gas fermentation, whereas with heat integration only 29% of this heat duty needs to be obtained by
combusting a fraction of the gasifier's H₂ product. Recovering the heat generated to fuel the CBB cycle removes the thermodynamic inefficiency of CO₂ fixation as a significant burden to the process techno-economics. Although lignin is an abundant and low-cost feedstock, its recalcitrant, complex structure makes its direct exploitation in fermentation challenging. The process engineering solution in Figure 7 is not only energy efficient but also provides an innovative solution via scH₂O gasification using renewable feedstocks such as lignin to produce chemicals. This study unlocks the promise of sustainable manufacturing using renewable feedstocks by combining the carbon efficiency of bio-catalysis with energy efficiency enforced through process engineering.

Limitations of the Study

In the design of a sustainable bio-manufacturing facility, capital cost is directly proportional to the productivity in the bioreactors, which impacts significantly on achieving favorable techno-economics. Although...
this work is the first to demonstrate the stable and continuous bio-manufacture of chemicals from CO$_2$ using *C. necator* as a carbon-efficient cell factory, productivity in gas fermentation will be limited by O$_2$ transfer constraints in light of H$_2$ flammability. Therefore, process intensification toward higher O$_2$ mass transfer remains an important continued area of research. Although this study demonstrated stable and continuous gas fermentation experimentally, the integration of gas fermentation with scH$_2$O gasification was verified through process simulation. Notably, process simulators, such as Aspen HYSYS, provide for rigorous simulation that enables effective process design. Although this study is the first to demonstrate the use of process engineering to overcome the techno-economic hurdle associated with the energy inefficiency of biological CO$_2$ fixation, this work will benefit from the future demonstration of this integrated, continuous process at large laboratory scale.

**Resource Availability**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Rajesh Reddy Bommareddy (rajesh.bommareddy@nottingham.ac.uk).

**Materials Availability**

Materials generated in this study are available from the Lead Contact under Material Transfer Agreement (MTA).

**Data and Code Availability**

The ScrumPy package of metabolic modeling tools (build OMICS$_{20,375}$) was used for all systems biology simulations (Poolman, 2006). Aspen HYSYS V11 (build 37.0.0.395) from Aspen Technologies, Inc. was used for all process simulations in this study. Experimental sensor and analytical data from shake flask and continuous fermentation experiments (please see Transparent Methods section) were processed in Microsoft Excel 2016. Graphical representations of the processed data were produced using MATLAB R2019b.
METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101218.

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AUTHOR CONTRIBUTIONS
Conceptualization: R.R.B., E.L., A.V.C.; Methodology: R.R.B., Y.W., and A.V.C; Software: Y.W., N.P., and A.V.C; Formal Analysis: R.R.B., N.P., and A.V.C; Investigation: R.R.B., Y.W., N.P., and A.V.C; Writing – Original Draft: R.R.B and A.V.C; Writing – Review & Editing: R.R.B., Y.W., N.P., M.H., N.P.M., E.L., and A.V.C.; Visualization: R.R.B, Y.W., and A.V.C.; Supervision: A.V.C.; Funding Acquisition: N.P.M. and A.V.C.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

A Sustainable Chemicals Manufacturing Paradigm

Using CO$_2$ and Renewable H$_2$

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SUPPLEMENTAL INFORMATION

TRANSPARENT METHODS

METABOLIC ENGINEERING

Gene selection

The biochemical network for the synthesis of BDO and IPA in the microbial cell factory, C. necator H16, is outlined in Figure 1A. The BDO biochemical pathway entails three enzymatic steps from pyruvate, where pyruvate is converted to 2-acetolactate by the feedback resistant 2-acetolactate synthase from Bacillus subtilis (ALS, alsS). Thereafter, 2-acetolactate is decarboxylated to stereospecific (R)-acetoin by acetolactate decarboxylase from Bacillus subtilis (ALDC, alsD). Finally, (R)-acetoin is reduced to BDO by a NADPH-specific secondary alcohol dehydrogenase (sADH, adh) from Clostridium beijerinckii. The isopropanol (IPA) pathway entails a four-step enzymatic conversion of two moles of acetyl-CoA to one mole of IPA (Figure 1A). Acetyl-CoA is converted to acetoacetyl-CoA by a β-ketothiolase from C. necator (phaA). Thereafter, acetoacetyl-CoA is hydrolysed to acetoacetate by a CoA-transferase from Helicobacter pylori (ctfAB, ctfAB) and decarboxylated to acetone by a decarboxylase from Clostridium acetobutylicum (ADC, adc). Finally, acetone is reduced to IPA by a NADPH-dependent secondary alcohol dehydrogenase from Clostridium beijerinckii (sADH, adh). The genes encoding for the enzymes of the BDO and IPA pathways are detailed in Table S4.

Synthetic biology

Growth media and heterotrophic cultures

Unless stated otherwise, lysogeny broth (LB) was used for routine cultivations of E.coli and C. necator, adding 15 [g/L] agar for solid media plates. Where required, antibiotic selection pressure at 300 [µg/mL] and 50 [µg/mL] kanamycin (kan') was used for C. necator and E.coli cultures respectively. For pLO3 vector based cultivations, 12.5 [µg/mL] tetracycline (Tc') was used for E.coli and C. necator. Shake flask cultivations were performed in 500mL baffled flasks with 50 [mL] working volume and incubated at 30 [°C] and 200 [rpm]. The minimum media recipe advocated by (Schlegel et al., 1961) was used for all microbial shake flask performance evaluations including 20 [g/L] fructose as carbon source. Shake flask evaluations were incubated for 72 [h].

Plasmid cloning & transformation protocols

The genetic constructs for the BDO and IPA pathways were codon optimised for expression in C. necator and synthetised using Invitrogen Geneart® gene synthesis (Table S5). All primers used in the study are listed in Table S1. DNA amplification was performed using Phusion U hot start
polymerase (NEB) with GC buffer for USER assembly. GC rich DNA amplifications were performed
using Phusion polymerase with green GC buffer (NEB). For routine DNA amplifications, DreamTaq
polymerase master mix (Thermo Scientific) was used. The pBAD promoter with the araC gene was
obtained from the plasmid, pCM291rfp. Ribosome binding sites (RBS) were taken from previous
studies as outlined in Table S2. Plasmids (see Table S3) were constructed with USER assembly kit
(New England Biolabs, NEB) and transformed into E.coli DH5α by heat-shock method. The
plasmids were extracted using mini-prep kit from NEB (Monarch®) and transformed into C. necator
by electroporation using a 0.2cm cuvette (Bio-rad), at 2.5 kV, 200Ω and 25μF using a GenePulser®
electroporation unit (Ausubel, 2003).

Chromosomal attenuation and integration protocol
For chromosomal attenuation and integration, suicide vectors (see Table S3) were transformed via
conjugation using E.coli S-17 cells carrying the desired vector and C. necator strains using the
protocol described by Lenz et al (Lenz and Friedrich, 1998). Suicide vectors (pLO3 based) with
flanking regions were created by overlap extension PCR using primers listed in Table S1. The BDO
operon was placed between 750bp homology arms, each flanking upstream of phaC1’s start codon
and downstream of phaB1’s stop codon, producing a suicide vector pLO3-CAB-BD2 (Table S3).
This vector was then used to knock-out the phaC1AB1 operon in H16 and knock-in the BDO operon,
creating the strain H16-CAB-BD2. The IPA operon was placed between 750bp homology arms each
flanking 750bp upstream of phaC1 start codon and phaC1 stop codon producing vector pLO3-CB-
IPA4. This vector was used to attenuate phaC1 in the H16-B strain (knockout of phaB1) and knock-
in the IPA operon upstream of phaA gene. Trans-conjugants were selected on minimal media plates
with 0.4 [%] (w/w) fructose and appropriate antibiotics. Knock-in and knock-off events for
integrating the operons were performed by two-step homologous recombination (tet’ and sacB
selection). The first recombination event, i.e. the first crossover, was confirmed on LB plates using
tetracycline as the selection marker. The second recombination step was carried out by inoculating
single colonies from the first crossover into low salt LB medium with 15 [%] (w/v) sucrose without
antibiotics overnight. Sucrose resistant colonies were plated on low salt LB agar plate with 15 [%]
(w/v) sucrose, and single colonies were selected for further screening. Colonies without antibiotic
resistance (tet’) were selected and successful integration was confirmed via PCR using primers
flanking the upstream and downstream regions of the chosen homologous sequences. Integration
was also confirmed using Sanger sequencing (Eurofins genomics GmbH).

GAS FERMENTATION

Gas fermentation media
Pre-cultures for bioreactor cultivations were prepared in tryptic soy broth (TSB) medium from a
single colony and incubated overnight at 200 [rpm], 30 [°C] in 500mL baffled flask with 100 [mL]
working volume. Modified DSMZ 81 medium was used for autotrophic bioreactor cultivations, where the vitamin solution and NaVO₃ were omitted. Metaphosphates were added instead of orthophosphates in the form of trisodium trimetaphosphate (Na₃P₃O₉) to avoid struvite precipitation. L-Arabinose was added at a final concentration of 1 [g/L] (0.1 [%] (w/v)) for culture induction.

**Bioreactor setup**

As depicted in Figure 3, continuous fermentations were performed in a DASGIP® bioreactor system (Eppendorf), using 1L capacity vessels with a 750 [mL] working volume, fitted with a headplate condenser chilled to 10 [°C]. The agitation system comprised two Rushton turbine impellers. Gas sparging was via two pin-hole, L-shaped spargers, separating the CO₂ and air feed from the H₂ feed. For continuous operation, the level in the bioreactor vessel was controlled (LIC100) using a conductivity probe positioned at an appropriate height in the vessel. The temperature was controlled (TIC100) at 30 [°C] using a chilled water feed. The pH was monitored by a Mettler Toledo pH probe, calibrated at pH = 4 and pH = 7 in standard pH buffers. The pH was controlled (AIC100) at a set point of 6.9 using 5 [%] (w/v) NH₃(aq). The dissolved oxygen (DO) concentration was monitored using an optical Mettler Toledo DO probe, calibrated in situ at atmospheric pressure in N₂ at 0 [%] pO₂ and in air at 100 [%] pO₂. The DO was controlled (AIC101) at micro-aerobic, steady state concentration through phosphate addition under phosphate limiting conditions. The off-gas outlet from the bioreactor was fed to an external foam trap bottle, fitted with an optical oxygen probe (VisiFerm, Hamilton), calibrated at 0 [%] (v/v) with N₂ and at 10 [%] (v/v) with 10 [%] (v/v) O₂ in N₂. The off-gas O₂ concentration was controlled (AIC102) at a non-flammable set point of 4 [%] (v/v) by controlling the air flow rate (FIC100) to the bioreactor. Guarding against a flammable atmosphere, a safety trip was programmed into the Dasware® software, which interlocked all the gases feeds should the off-gas O₂ concentration rise above 5 [%] (v/v), flooding the bioreactor with N₂.

The initial agitation speed (SIC100) was set at 400 [rpm] and stepped to a maximum agitation of 1600 [rpm] approximately 24 [h] after inoculation. CO₂ was fed via a mass flow controller (FIC101) at a rate of 1.35 [L/h]. H₂ was fed via mass flow controller (FIC102) at a rate of 35.1 [L/h]. The mass flow rates for CO₂, H₂ and Air mass flow controllers were calibrated using a M13 mini CORI-FLOW Coriolis Mass Flow Meter from Bronkhorst, ranged between 0 – 50 [g/h]. The Nutrient Feed media, containing 0.1 [%] (w/w) arabinose as inducer, was fed at 7 [mL/h] via a peristaltic pump. The off-gas from the external foam trap bottle was connected to a local exhaust ventilation (LEV) unit and the whole bioreactor setup was placed in the LEV with polycarbonate doors to ensure a ventilated enclosure.
**ANALYTICAL METHODS**

**Liquid fraction analyses**

Supernatants from the shake flask and fermentation cultivations were obtained by centrifuging culture samples for 5 [min] at 13000 [rpm]. Fructose, pyruvate, acetoin, meso-2,3-butanediol, R,R-2,3-butanediol, S,S-2,3-butanediol, acetone, acetate, succinate and isopropanol were analysed using an HPLC with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), equipped with UV and RI detectors. The flow rate of the 5 [mM] H$_2$SO$_4$ mobile phase was set at an isocratic 0.5 [mL/min] with a column temperature of 50 [°C]. Quantifications were performed from the standard curves obtained using standards purchased from Sigma Aldrich.

Dry cell weight (DCW) measurement was used as primary method for biomass concentration. DCW was determined by centrifuging 1 [mL] culture in a pre-weighted 2mL Eppendorf tube. After washing once with distilled water, the pellet was dried for 48 [h] at 100 [°C] and weighed. Optical density measurement was used as secondary method for biomass concentration, quantified using a spectrophotometer at an optical density of 600 [nm]. A correlation factor of 1 OD$_{600}$ = 0.30 ± 0.03 [g/L] biomass was determined with an R$^2$ = 0.96.

**Gas fraction analyses**

Off-gas analyses from fermentation were undertaken in-line after the foam trap using a multiplexed Raman Laser Analyzer from Atmospheric Recovery Incorporated. The span for the off-gas analyser was calibrated with two high specification gas mixtures from BOC Gases, containing ultra-high purity (UHP) argon as the background gas. The two gas mixtures were (1) 40 [%] (v/v) H$_2$ in UHP argon, and (2) 4 [%] (v/v) O$_2$, 50 [%] (v/v) N$_2$ and 3 [%] (v/v) CO$_2$ in UHP argon. The zero calibration was undertaken in UHP purity argon. The off-gas analyser was set to sample for 20 [s] from each fermenter in an 80 [s] cycle at a sample flow rate of 280 [mL/min].

**SYSTEMS BIOLOGY AND PROCESS SIMULATION**

**Systems Biology biochemical network simulation**

The ScrumPy metabolic modelling software package (Poolman, 2006) was used to simulate carbon fluxes using a genome scale model for C. necator (Unpublished Data), which advances on the existing model proposed by Park et al (Park et al., 2011). The cell’s ATP maintenance requirement for autotrophic growth was set at 10 [mmol/(gDCW-h)] and 3 [mmol/(gDCW-h)] for heterotrophic growth. The BDO pathway genes were added to the genome scale model for wild-type C. necator H16 as per Figure 1A. For the simulations using guaiacol as sole energy and carbon source, the cytochrome P450 aromatic O-demethylase characterised by Mallinson et al (Mallinson et al., 2018) was incorporated into the genome scale model. Carbon uptake rates for the carbon source, either
CO$_2$ or guaiacol, and biomass growth rates were constrained to align with fermentation data. The objective function for Flux Balance Analysis (FBA) was to maximise the BDO production in light of the overexpressed BDO pathway.

**Aspen HYSYS process simulations**

The process simulator, ASPEN HYSYS V11, was used to rigorously model the heat integration of gas fermentation with scH$_2$O gasification through a heat pump thermal cycle. The Lee-Kesler-Plocker equation of state was used to model the thermodynamic properties of the process fluids, given it represents the most accurate enthalpy model for high pressure gases. The scH$_2$O gasification reactor was modelled as a plug flow reactor using the pseudo first order, Ni-catalysed kinetic rate constant proposed by DiLeo et al (DiLeo et al., 2007). The bioreactor was modelled as a conversion reactor using the experimentally determined reaction stoichiometry for CO$_2$ & H$_2$ and the FBA simulation stoichiometry for the guaiacol bioreactor. Similarly, the combustion chamber was modelled as a conversion reactor, assuming total conversion of the H$_2$ and O$_2$ to H$_2$O. Compressors were modelled having a single stage with an adiabatic efficiency of 75 [%]. Turbines were modelled as having an isentropic efficiency of 75 [%]. Heat exchangers were modelled as single pass shell-and-tube, maintaining a minimum approach temperature of 10 [°C]. Given the high accuracy of the Lee-Kesler-Plocker equation of state, ASPEN HYSYS was also employed to estimate the volatile product fraction for the BDO and IPA continuous gas fermentations.
### Table S1. Sequences of primers. Related to Figure 1B.

| Primer   | Sequence (5’ ----> 3’)                                                                 |
|----------|----------------------------------------------------------------------------------------|
| U-ara-F  | gggaaagUaacgttatgacaacttgacggctac                                                  |
| U-ara-R  | atatctccUtcttaaaaagatcttttgaatcc                                                   |
| U-alsS-F | agggataUacatatgaccaaggccaccaaggaacag                                                |
| U-alsS-R | atgttaacUtctctttacgtacacacgccttgcctctcatcgc                                        |
| U-alsD-F | agttaccaUgaagcgagtcgaacatccag                                                      |
| U-alsD-R | aggttgUcctcttttctgatctattcggcagccagccttg                                           |
| U-sADH-F | acaaccaUgaagggcttcgcagacc                                                          |
| U-sADH-R | ggagacaUccatagtcagagctaccaaggcagcc                                                 |
| Aco-up-sacl-F | ttatgactctactacggcctcaacggccg                                                         |
| Aco-dn-xbal-R | ttatctaggctcaggttgagatccgc                                                       |
| Aco-ov-spel-F | ggagacagggcaatgggacacactagtcagtcgggcgtgamgctg                                      |
| Aco-ov-spel-R | ggagtccagccgccccagatcaggtgctggccccattcctgcc                                          |
| phaC-up-F | acgcggcgtgaacaggtc                                                                |
| phaB-dn-R | tgctctcatgctcgcgtgcatctacg                                                         |
| phaC-up-sacl-F | ttatgactcagcgccgctctactctcactc                                                      |
| phaB-dn-pacl-R | attatattaattaactgatctagttgctcagatgccc                                              |
| phaCB-ov-spel-F | acgcggagagacagacaaatcactaggcttcagggcctggtttcaaccag                                       |
| phaCB-ov-spel-R | ctgtttgaacagggccggcagcttaggacatgtctgtgtctgtctgcc                                   |
| U-ctfAB-F | agggataUacatatgaccaaggtgatcacc                                                       |
| U-ctfAB-R | atgttaacUctctctttcagtcagacacccctgagaactcgt                                         |
| U-adc-F  | agttaccaUgaagcgagtcgaacatccag                                                        |
| U-adc-R  | atgttgUacatggtctggtctcagttcagatgatctcag                                          |
| phaC-dn-pacl-R | attatattaattaactgatctagttgctcagattcacc                                             |
| phaC-dn-R | tgctctcatgctcgcgtgcatctacg                                                         |
| phaC-ov-spel-F | ggccagacagagacaatcactatggcgactagtaaggcagcttgcatgtcgactgc                              |
| phaC-ov-spel-R | gcacatctatgccaagctactcctctcttactgtgccattttgattttgacttcctgcc                        |
| phaB1-up-sacl-F | ttatgactcagacacgccgaggc                                                            |
| phaB1-dn-xbal-R | attatctagcctggatgctttttctcag                                                       |
| phaB1-ov-spel-F | acgcggagagacagacaaatcactaggcttcagggcctggtttcag                                         |
| phaB1-ov-spel-R | gaaccagggcggcagcttagtccacacccctgtgatctgcc                                          |
| phaB1-up-F  | tcaacgccggagacgtggtgc                                                                |
Table S2. Ribosome binding sites. Related to Figure 1B.

| RBS  | RBS sequence                     | Reference                      |
|------|----------------------------------|--------------------------------|
| RBS1 | ttaaggaaggagatatatacatATG        | (Bi et al., 2013)              |
| RBS2 | cgtacgtaaaggaggaaggattacAG      | (Li and Liao, 2013)            |
| RBS3 | aaagggagaccaaccAG               | (Grousseau et al., 2014)       |

Table S3. Strains and plasmids used in this study. Related to Figure 2.

| Cupriavidus necator strains | Genotype or description                             | Reference or source               |
|-----------------------------|----------------------------------------------------|----------------------------------|
| H16                         | wild-type                                          | DSM 428, ATCC 17699              |
| H16-CAB                     | H16 with in-frame deletion of phaC1AB1 operon      | This study                       |
| H16-CAB-Aco                  | H16 with in-frame deletion of phaC1AB1 and acoXABC operons | This study                       |
| H16-CAB-Aco-BD-2             | CAB-Aco with integrated 2,3-BDO operon with NADPH-dependent alcohol dehydrogenase | This study                       |
| H16-CAB-Aco-p-BD-2           | CAB-Aco with plasmid pBBR-BD-2                     | This study                       |
| H16-CB-IPA-4                 | H16-CB with integrated IPA-4 pathway               | This study                       |
| H16-CB-p-IPA-4               | H16-CB with pBBR-IPA-4                            | This study                       |
| H16-CB                      | H16 with in-frame deletion of phaC1 and phaB1      | This study                       |
| H16-B                       | H16 with in-frame deletion of phaB1                | This study                       |

E.coli strains

| DH5α                         | fhuA2 Δ(argF-lacZ)U169 phoA glnV44 F80 Δ(lacZ)M15 gyrA96 recA1 | Invitrogen                      |
|------------------------------|---------------------------------------------------------------|--------------------------------|
| S17-1                        | relA1 endA1 thi-1 hsdR17 recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome | Invitrogen                      |

Plasmids

| pBBR1MCS-2-USER              | Expression vector, Kan’                                     | (Alagesan et al., 2018)         |
| Cupriavidus necator strains | Genotype or description | Reference or source |
|-----------------------------|-------------------------|---------------------|
| pCM291rfp                  | Source of pBAD promoter with *araC* gene | (Bi et al., 2013) |
| pLO3                      | Suicide vector, Tc′, *sacB*, RP4 ori, ColE1 ori | (Lenz and Friedrich, 1998) |
| pLO3-CB                   | pLO3 with 750 bp upstream region of *phaC*1 and 750 bp downstream region of *phaB*1 | This study |
| pLO3-C                    | pLO3 with 750 bp upstream region of *phaC*1 and 750 bp downstream region of *phaC*1 | This study |
| pLO3-B                    | pLO3 with 750 bp upstream region of *phaB*1 and 750 bp downstream region of *phaB*1 | This study |
| pBBR-BD-2                 | pBBR1MCS-2-USER with pBAD-ALS-ALDC-sADH | This study |
| pLO3-CAB-BD-2             | pLO3 with 750 bp upstream region of *phaC*1-pBAD-ALS-ALDC-sADH and 750 bp downstream region of *phaB*1 | This study |
| pLO3-acoXABC-KO           | pLO3 with 850 bp upstream region of *acoX* and 900 bp downstream region of *acoC* | This study |
| pBBR-IPA-4                | pBBR1MCS-2-USER with pBAD-ctfAB-ADC-sADH | This study |
| pLO3-C-IPA-4              | pLO3 with 750 bp upstream of *phaC*-pBAD-ctfAB-ADC-sADH and 750 bp downstream region of *phaC*1 | This study |

**Table S4.** Pathway gene selection. Related to Figure 1B.

| Pathway       | Gene | Enzyme                      | Host Origin          | Accession number | Reference                      |
|---------------|------|-----------------------------|----------------------|------------------|--------------------------------|
| BDO pathway   | *alsS* | 2-acetolactate synthase     | *Bacillus subtilis*  | EnsemblBacteria: BSU36010 | (Yan et al., 2009)            |
| *genes*       | *alsD* | acetolactate decarboxylase  | *Bacillus subtilis*  | EnsemblBacteria: BSU36000 | (Yan et al., 2009)            |
|               | *adh*  | NADPH-specific secondary alcohol dehydrogenase | *Clostridium beijerinckii* | GenBank: AAA23199.2 | (Yan et al., 2009)            |
| IPA pathway genes | ctfAB | CoA-transferase | *Helicobacter pylori* | GenBank: AJ000086.1 | (Corthésy-Theulaz et al., 1997) |
|-------------------|-------|----------------|----------------------|---------------------|----------------------------------|
|                   |       |                |                      |                     |                                  |
| *adc*             | acetoacetate decarboxylase | *Clostridium acetobutylicum* | EnsemblBacteria: CA_P0165 | (Grousseau et al., 2014) |
| *adh*             | NADPH-specific secondary alcohol dehydrogenase | *Clostridium beijerinckii* | GenBank: AAA23199.2 | (Yan et al., 2009) |

**Table S5.** Condon optimised sequences. Related to Figure 1B.

| Gene | Sequence |
|------|----------|
| *alsS* | atgaccaagcccccaagcaaggaacgagtcgctgctaagaatcgccggccagacttggtggtgactcttggtggaaccgggctggtgcggtgtccttcgcagctgcgcggccagaaggaccagccggtcagccaaatctaccaaggtgtcgaccatgacctcgctgctggacggcgtgtacgacggcgacttcgagctgtcggagatcccgaagtacggcgatttcggcatcggcaccttcacaacaagctggatggcgagctgatcggcttcgacggcgagttctaccgcctgcgctcggatggcaccgccacgccggtgcagaacggcgatcgcagcccgttctgctcgttcaccttcttcaccccggacatgacccacaagatcgacgccgaagatgaccgcgcgaggaacttcgagaaggaaatcaactcgatgctgccgtcgcgcaacctgttctacgccatccgcatcgacggcctgttcaagaaggtgcagacccgcaccgtggaactgcaggaaaagccctacgtcccgatggtggaagccgtcaagacccagccgatcttcaacttcgacaacctgcgcggcaccatcgtgggcttcctgacgccggcctacgccaacggcatcgccgtgtcgggctaccatctgcactttacgcacg |
| gene | sequence |
|------|----------|
| adh  | aaggccgcaacagcggcggccacgtgttcgactacgtgctggaagattgcaccgtgaccatctcgcagaagatgaacatgaacctgcgcctgccgaacaccgccgatttcttcaacgccaacctggacaacccggacttcgccaaggacatcgaaaccaccgagggctgcgcgaatga |
| ctfAB| atgaaacaggtgatcaagcgcgaactttgcgtgaccaacccggtgcggctttggcctgtgcggcatcccggaatacgccatcgactacatctacaagaagggcatcaaggacctgatcgtggtgtcgaacaactgcggcgtgacgacttcggcctgggcatcctgctggaaaagaagcagatcaagaagatcatcgccagctacgtgggcgagaacaaaatcttgagtcgcagatgctgaacggcgagatcggaggtggtgctgaccccgcagggcaccctggccgaaaatctgcgccccggcggcgcgggcatccccgcctactaccccgaccggcgtgggcacgctgatcgcccagggcaaggaatcgcgcgagttcaacggcaaaggaatacatcctggaacgcgccatcaccggcgactacggcctgatcaaggcctacaagtcggacaccctgggcaacctggtgttccgcaagaccgcgcgcaacttcaacccgctgtgcgccatggccgccaaaatctgcgtggccgaggtggaagagatcgctgccggcgggcgaactggacccggacgagatccatctgccgggcatctacgtgcagcatatctacaagggcgagaagttcgagaagcgcatacgaaaaagatcaccacgcgctcggccaaatgagggaagccatcatcaagcgcgccgccaaggaactgaaggaaggcatgtaagctcaatctgggcatcggcctgccgacgctggtcgccaatgaggtgtcgggcatgaacatcgtgttccagtcggagaacggcctgctgggcatcggcgcgtatccgctggaaggctcggtggacgccgacctgatcaatgccggcaaggaaaccgtgacggttcgccgagttccgagccctcgttcttcaactcggccgactcgttcgccatgatccgcggcggccatatcgacctggcgatcctgggcggcatggaagtgtcgcagaacggcgacctggccaactggatgatcccgaagaagctgatcaagggcatgggcggcgccatggacctggtgcatggcgcgaagaaggtcatcgtgatcatggaacactgcaacaagtacggcgagagcaaggtgaagaaggaatgctcgctgacgctgaccgggaagggcttcgccatgctgggcatcaacaagaatctgcttgcttgcttggtgccgagctgcaggaaggcgtcagcctggaccaggtgcgcgaaaagaccgaggccgagttcgagtcctgtga |
| adc  | atgcggctagcataacgcgagaagccgacaccatctcgtgatccggcggctttggccttgctgcaagctgcaggaaggcgtcagcctggaccaggtgcgcgaaaagaccgaggccgagttcgagtcctgtga |
**Table S6.** Flux Balance Analysis (FBA) systems biology prediction for autotrophic and heterotrophic synthesis of BDO using CO$_2$ & H$_2$ and guaiacol as sole energy and carbon sources respectively. The nominal experimental biomass growth rate of 0.0164 [h$^{-1}$] was use for both FBA simulations. Related to Figure 6 and Table 2.

| Cell sources and sinks | Unit       | Guaiacol as sole energy and carbon source | CO$_2$ and H$_2$ as sole energy and carbon source |
|------------------------|------------|------------------------------------------|--------------------------------------------------|
| **Sources**            |            |                                          |                                                  |
| CO$_2$                 | mmol/(gDCW-h) |                                          | -3.0                                             |
| H$_2$                  | mmol/(gDCW-h) |                                          | -22.16                                           |
| Guaiacol               |             | -0.43                                    |                                                  |
| O$_2$                  |             | -2.03                                    | -7.2                                             |
| P$_i$                  |             | -0.04                                    | -0.04                                            |
| SO$_4^{2-}$            |             | -0.003                                   | -0.003                                           |
| NH$_3$                 |             | -0.17                                    | -0.17                                            |
| **Sinks**              |            |                                          |                                                  |
| BDO$^a$                | mmol/(gDCW-h) | 0.091                                    | 0.582                                            |
| H$_2$O                 | mmol/(gDCW-h) | 0.95                                     | 18.94                                            |
| CO$_2$                 |             | 1.974                                    |                                                  |
| Biomass                | [h$^{-1}$]  | 0.0164                                   | 0.0164                                           |

$^a$BDO is 2,3-butanediol.
Table S7. Summary of ASPEN HYSYS process simulations using a guaiacol feed basis of 1000 [kg/h] for the process flowsheets of Figure 9 and Figure 8, scaling the bioreactors for the guaiacol and CO₂ & H₂ fermentations using a design O₂ transfer rate of ~225 [mmol/(L-h)]. Related to Figures 8 and 9.

| Sources and sinks | Unit                  | Guaiacol as sole energy and carbon source | CO₂ and H₂ as sole energy and carbon source |
|-------------------|-----------------------|------------------------------------------|--------------------------------------------|
| Guaiacol Feed Rate | [kg/h]                | 1000                                     | 1000                                       |
| Gas Uptake Rates  |                       |                                          |                                            |
| O₂                | [mmol/(L-h)]          | 223                                      | 227                                        |
| CO₂               | [mmol/(L-h)]          | 82                                       |                                            |
| H₂                | [mmol/(L-h)]          | 669                                      |                                            |
| Bioreactor Volume | [m³]                  | 200                                      | 100                                        |
| 2,3-butanediol Productivity |          |                                          |                                            |
| Specific Productivity | [(kg BDO)/(m³-h)] | 0.79                                     | 1.48                                       |
| Concentration a  | [g/L]                 | 30                                       | 30                                         |
| Biomass           |                       |                                          |                                            |
| Growth rate       | [h⁻¹]                 | 0.017                                    | 0.016                                      |
| Dry Cell Weight   | [g/L]                 | 102                                      | 27                                         |
| Operating costs   |                       |                                          |                                            |
| Electricity demand | [kW]                  | 2382                                     | -566                                       |
| Cooling water     | [kW]                  | 7702                                     | -                                          |

* Controlled via microfiltration membrane.
Figure S1. (A) BDO tolerance of *C. necator* H16 wild type, expressed as growth rate versus BDO concentration. Beyond a BDO concentration of 30 [g/L], the growth rate becomes significantly impaired. Related to Figure 1A.
Figure S2. Methodology for calculating (1) gas uptake rates, (2) dilution rate at steady state and (3) product and by-product carbon flux for continuous, autotrophic fermentation. Related to Table 2 and Figures 4 and 5.
Figure S3. Monte Carlo simulations incorporating all sensor, analysis and calibration standard uncertainty into the respective calculated fermentation data. 90% confidence limits were determined from the cumulative probability curves. Related to Figure 4 and 5.
Figure S4. Biochemical network outlining the synthesis of (R,R)-2,3-butanediol from the lignin model compound, guaiacol, in the microbial cell factory, C. necator H16. Genes overexpressed to allow (R,R)-2,3-butanediol synthesis are contained in grey text boxes, whilst attenuated genes are contained in red text boxes. Guaiacol is demethylated to catechol and catabolised via the 3-oxoadipate pathway to succinyl-CoA and acetyl-CoA. Pyruvate is produced from malate via malic enzyme as the metabolite precursor to BDO synthesis. Related to Figures 8 and 9.