Maximizing the conversion of biogenic carbon feedstocks into chemicals and fuels is essential for fermentation processes as feedstock costs and processing is commonly the greatest operating expense. Unfortunately, for most fermentations, over one-third of sugar carbon is lost to CO₂ due to the decarboxylation of pyruvate to acetyl-CoA and limitations in the reducing power of the bio-feedstock. Here we show that anaerobic, non-photosynthetic mixotrophy, defined as the concurrent utilization of organic (for example, sugars) and inorganic (for example, CO₂) substrates in a single organism, can overcome these constraints to increase product yields and reduce overall CO₂ emissions. As a proof-of-concept, Clostridium ljungdahlii was engineered to produce acetone and achieved a mass yield 138% of the previous theoretical maximum using a high cell density continuous fermentation process. In addition, when enough reductant (that is, H₂) is provided, the fermentation emits no CO₂. Finally, we show that mixotrophy is a general trait among acetogens.
The production costs for most chemicals via microbial fermentation are currently high compared to oil-derived products primarily because of operating costs associated with feedstock and feedstock processing. Consequently, first and second generation bioproduct manufacturing processes are economically challenged, particularly in light of recent low oil prices. One way to mitigate high feedstock cost is to maximize conversion into the bioproduct of interest. This maximization, though, is limited because of the production of CO₂ during the conversion of sugar into acetyl-CoA in traditional fermentation processes. Acetyl-CoA is a central building block and a link between glycolysis and almost all downstream metabolic pathways and serves as a focal point for the production of biofuels and industrial chemicals by microbial fermentations. However, the ability to achieve metabolically efficient production of acetyl-CoA is hindered by energetic requirements and biochemical pathway constraints, requiring the production of CO₂ for every acetyl-CoA produced from glycolysis. Thus, one-third of all carbon in the feedstock is lost to CO₂, resulting in maximum carbon conversion of 67% at best, and lower in actuality due to cell mass creation, cell maintenance needs and other constraints. It was previously demonstrated that a synthetic, non-oxidative glycolysis (NOG) pathway enables the stoichiometric conversion of certain sugars to acetyl-CoA; however, NOG does not generate adenosine triphosphate (ATP) without converting acetyl-CoA into acetate, and consumes all reducing equivalents (that is, NAD(P)H) produced from sugar. Consequently, NOG-based product yields are limited by both ATP and NAD(P)H when producing metabolites that are more reduced, on a carbon basis (that is, carbon degree of reduction), than the feedstock consumed.

A second, alternative approach that stoichiometrically converts sugar to acetyl-CoA is an anaerobic, non-photosynthetic mixotrophic fermentation (here referred to as mixotrophy). Mixotrophy is defined as the concurrent utilization of organic (for example, sugars) and inorganic (for example, CO₂, CO and H₂) substrates for growth and metabolism. As reviewed, the Wood–Ljungdahli Pathway (WLP), the carbon fixation pathway employed by acetogens to convert CO₂:H₂, CO or other C1 feedstocks into acetyl-CoA, is particularly well-suited for mixotrophy because it exhibits a low ATP requirement relative to other carbon fixation pathways and requires the exact amount of NAD(P)H generated through glycolysis to fix two molecules of CO₂ into one acetyl-CoA. Thus, one mole of hexose sugar yields three moles of acetyl-CoA and one mole of ATP through WLP-driven mixotrophy. With their ability to utilize gases through the WLP and a broad array of other carbohydrate substrates, acetogens are an ideal host organism for mixotrophy.

The amount of CO₂ re-assimilated with mixotrophy depends upon the degree of reduction of the desired metabolite (product). The more reduced the product, the less CO₂ can be re-assimilated, because NAD(P)H is directed towards product formation rather than CO₂ fixation. However, this reducing equivalent deficiency can be overcome through H₂-enhanced mixotrophy, whereby sufficient H₂ is exogenously provided to fully recapture the CO₂ lost in glycolysis. To avoid CO₂ emissions associated with H₂-production, electrolysis of water powered by solar, wind or hydroelectricity would be a preferred source and has achieved a level of maturity and success. Alternatively, syngas can be added to sugar fermentation to provide the necessary reducing power and carbon. As reviewed, WLP gas-only fermentation is an ATP-limited process commonly requiring production of acetate from acetyl-CoA, for which acetogens receive their name, to generate sufficient ATP for cell growth and maintenance. Syngas-enhanced mixotrophy mitigates this challenge by supplying cells with abundant ATP through glycolysis.

In this study, we demonstrate the ability of a broad range of acetogenic organisms to conduct mixotrophy and H₂- or syngas-enhanced mixotrophy without carbon catabolite repression (CCR), thus enabling sugar to metabolite yields that are not theoretically possible through heterotrophic (that is, traditional) fermentation. Additionally, we demonstrate the ability to produce reduced products without the need for significant co-production of acetate, as is commonly witnessed in autotrophic fermentations. Moreover, we show that sugar can be stoichiometrically converted to reduced products with nearly no CO₂ production from glycolysis. Last, we demonstrate the utility of mixotrophy in acetone production using a genetically-engineered acetogen (Clostridium ljungdahlii, abbreviated as CLJ). Acetone, a commodity petrochemical currently produced through the cumene process, has a world market on the order of six million metric tons per year which is valued at nearly eight billion US dollars. This makes acetone an attractive target for renewable, biochemical production. However, the high cell density continuous fermentation system under mixotrophic conditions, we could achieve 138% of the theoretical, heterotrophic fermentation maximum, which is 92% of the theoretical mixotrophic maximum, and this was accomplished with volumetric productivities over 2 g l⁻¹ per hour with acetone titers greater than 10 g l⁻¹.

**Results**

**Concurrent utilization of gas and sugar.** A key concern regarding the implementation of mixotrophy (Fig. 1a) is the possibility of CCR of the WLP in the presence of a preferred sugar substrate, as has been shown for two acetogens: Clostridium aceticum and Blautia cocoides GA-1 (ref. 13). In contrast, Eubacterium limosum (ELM) and Acetobacterium woodii were found to concurrently utilize glucose and methanol and fructose and CO₂:H₂, respectively. To investigate if CCR occurs in CLJ, an important industrial strain, CLJ was grown on C₁₂-fructose and a C₁₃-labelled syngas mixture (13CO₂:13CO₂:H₂:N₂, 55:10:20:15) and interrogated at the metabolite, transcript and protein levels. CLJ incorporated a surprisingly large percentage of 13C-labeling, since it is also derived from glucose through the fermentation (Fig. 1d). Though it was not directly measured, ethanol, for both CLJ and CAU, should have a similar measured, ethanol, for both CLJ and CAU, should have a similar
**Figure 1 | The concept of mixotrophy and its demonstration.** (a,b) Different modes of fermentation are shown as an abbreviated metabolic network (a) and block flow diagrams (b). Heterotrophy (case I): hexose is consumed and CO₂ and potentially H₂ are produced. Mixotrophy (case II): hexose is consumed and excess reducing equivalents are used to fix endogenously produced CO₂; any unconsumed CO₂ is released from the process. H₂-enhanced mixotrophy (case III): hexose along with H₂ are fed to the microorganism and no CO₂ is released. Syngas-enhanced mixotrophy (case IV): hexose and CO₂:CO:CO₂:H₂ are fed to the microorganism. Depending on the composition of the syngas and the metabolite of interest, CO₂ may still be released from the process. Dashed lines indicate potential pathways or products. (c,d) ¹³C-labelling fermentation profiles of CLJ (c) and CAU (d) during syngas-enhanced mixotrophy. Fructose (black line) consumed and metabolites produced during fermentation in the presence of a syngas mixture (¹³CO, ¹³CO₂, H₂ and N₂). The percentage of acetate labelled with ¹³C is shown in light blue for each time point. The s.d. of two biological replicates is shown in black error bars.
allows for all excess reducing equivalents to be used for CO₂ fixation. In addition, acetone is an important commodity chemical and a feedstock for poly(methyl methacrylate) production. Acetone is not a natural metabolite of any known acetogen, but recombinant production has been engineered into *C. aceticum*¹⁸,¹⁹ and *A. woodii*²⁰. However, both studies focused primarily on autotrophic growth where significant amounts of acetate were still produced, and although acetone titers and volumetric productivities demonstrate proof-of-principle, results lack industrial relevance. Additionally, an inducible acetone-producing strain of CLJ was previously constructed²¹, though a recent publication²² has questioned whether isopropanol was actually produced instead of acetone because of the native secondary alcohol dehydrogenase (SADH) activity of CLJ²².

The SADH in CLJ is part of the 2,3-butanediol production pathway to convert acetoin into 2,3-butanediol and works in tandem with a 2,3-butanediol dehydrogenase (2,3-BDH; refs 22,23) (Fig. 2a). Importantly, this SADH was shown to convert exogenously added acetone into isopropanol²². To prevent this conversion, we deleted the SADH gene (CLJU_c24860) from the chromosome using a homologous recombination approach. We then constructed the acetone-producing plasmid pTCtA, consisting of a thiolase, a CoA-transferase, and an acetoacetate decarboxylase, and introduced it into the deletion strain CLJ ΔSADH. The resulting strain, CLJ ΔSADH (pTCtA), produced mostly acetone and acetate but also 3-hydroxybutyrate (3-HB) and trace amounts of isopropanol. We suspect 3-HB and isopropanol are still being produced to minor degrees because of the endogenous activity of 2,3-BDH, which is acting upon the intermediate acetoacetate to produce 3-HB or upon acetone to produce isopropanol (Fig. 2a).

After 168 h of mixotrophic growth, the total molar yield of CLJ ΔSADH (pTCtA) was 82% (Fig. 2b) with acetone being the primary metabolite at 1.73 g l⁻¹, followed by acetate at 1.54 g l⁻¹ and 3-HB at 0.29 g l⁻¹ (Fig. 2c, Supplementary Table 1). The mass yield of acetone was 34wt% and the mass yield of total acetone pathway products (acetone + 3-HB) was 37wt%. These mass yields are greater than the theoretical maximum acetone yield of 32wt% under standard heterotrophic conditions, demonstrating the ability to improve mass yields of products with mixotrophy. However, the total mass yield of acetone pathway products (37wt%) is only 80% of the theoretical maximum under mixotrophic conditions. We, therefore, investigated a high cell-density continuous fermentation process for CLJ ΔSADH (pTCtA) to obtain higher acetone yields.

**High cell density continuous fermentation.** A potent approach to further improve product (metabolite) yields, minimize biomass formation, reduce ATP demand and increase the availability of reduction energy (available electrons) is through continuous fermentation with cell recycle. Cell recycle can also increase cell density, which has the potential to increase volumetric productivity²⁴. To test this, we setup a high cell density continuous fermentation apparatus that fully retained cells until reaching an optical density at 600 nm (OD₆₀₀) of 35–60 (Fig. 3a), which corresponds to ~10–18 g l⁻¹ cell dry weight. As shown in Fig. 3a, the targeted cell densities were reached ~100 h after cell retention was started, at which point a harvest was implemented to maintain a constant cell density. Also at this point, the maximum acetone pathway (that is, acetone, 3-HB, and isopropanol) titer was achieved of 12.7 g l⁻¹ with concurrent acetate titers of 2.5 g l⁻¹. A minor amount of ethanol

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**Figure 2 | Metabolic engineering of CLJ to demonstrate mixotrophic production of acetone.** (a) Metabolic pathways downstream of pyruvate for the native and engineered CLJ. Native metabolites and enzymes are shown in black, and heterologous enzymes along with non-native metabolites are shown in blue. Integration with the WLP is shown through light blue arrows. The gene deletion is shown in red. PFOR, pyruvate:ferredoxin oxidoreductase; ALS, acetyl-CoA synthase; ALDC, acetolactate decarboxylase; AADC, acetoacetate decarboxylase; 23BDH, 2,3-butanediol dehydrogenase; SADH, secondary alcohol dehydrogenase; AK, acetate kinase; PTA, phosphotransacetylase; AAD, alcohol:aldehyde dehydrogenase; THL, thiolase; CoAT, CoA-transferase; AADC, acetoacetate decarboxylase. (b,c) Product profiles of CLJ ΔSADH (pTCtA) under mixotrophy and H₂-enhanced mixotrophy. Total molar yields (b) and product distributions (c) are shown. The s.d. of three biological replicates is shown in black error bars.
**Mixotrophy is a general trait of acetogens.** With the successful demonstration of mixotrophy in CLJ and CAU, we wanted to examine if mixotrophy was a more general trait among acetogens. Therefore, we tested two additional acetogens, *Moorella thermoacetica* (MTA) and ELM, for mixotrophy. ELM was previously shown to consume both glucose and methanol\(^1\), the latter being utilized through the methyl or ‘Eastern’ branch of the WLP\(^6\). Here we wanted to test for concurrent sugar and gas utilization. All four strains (CLJ, CAU, MTA and ELM) were tested under both mixotrophic conditions and syngas-enhanced mixotrophic conditions. In addition, a heterotrophic control was prepared with the anaerobic bacterium *C. acetobutylicum* (CAC), which is related to many acetogens but has no native carbon fixation pathway. Under mixotrophy, all acetogens achieved carbon molar yields greater than the 67% theoretical maximum yield (\(\text{C}_{\text{product}} \text{moles} / \text{C}_{\text{hexose consumed}} \text{moles}^{-1}\)) that can be achieved without carbon fixation (Fig. 4a). The carbon yields for CAU and ELM were less than those for CLJ and MTA, presumably because CAU and ELM produced a larger percentage of reduced products (ethanol for CAU and butyrate for ELM, Fig. 4b), thus reducing the amount of \(\text{CO}_2\) that can be fixed. Under syngas-enhanced mixotrophic conditions, the apparent carbon molar yields from sugar, as shown in Fig. 4a, for all acetogen species increased beyond the mixotrophic yields. In addition, the syngas mixture affected the product profiles with all strains, with more reduced products now being favoured (Fig. 4c). This shift occurs because of the added reducing power of the syngas mixture (55% CO and 20% \(\text{H}_2\)), which the cells are able to utilize to produce more reduced products. While acetate was still the primary metabolite, reduced products, like ethanol, over less reduced products, like acetone. This caused a shift in the metabolite profile, with the primary metabolite now ethanol at 2.42 g l\(^{-1}\) or 1.96 g l\(^{-1}\), for 20% and 40%, respectively, and acetone being reduced to 1.02 g l\(^{-1}\) or 0.81 g l\(^{-1}\), respectively (Supplementary Table 1). Nevertheless, these data demonstrate the power of this technology to enable complete carbon utilization. Directing the carbon to the desirable product will require further strain engineering to eliminate the formation of undesirable products and using only the necessary amount of \(\text{H}_2\) needed to achieve the zero \(\text{CO}_2\) loss goal.

**Mixotrophy benefits for other metabolites.** To highlight the potential of mixotrophic fermentations, we calculated the theoretical maximum yield for many metabolites of interest via mixotrophy and \(\text{H}_2\)-enhanced mixotrophy (Fig. 5a). Yield improvements are modest for more reduced products, such as ethanol and n-butanol, while yield improvements are significant for less reduced products including carboxylic acids. Generally, the ratio of mixotrophic to heterotrophic product yield is inversely proportional to the ratio of NAD(P)H to acetyl-CoA required.
to produce a given chemical (Supplementary Table 2). Accordingly, the NAD(P)H to acetyl-CoA ratio can be used to quickly determine the potential yield improvement from mixotrophy for most metabolites with acetyl-CoA as a precursor (Table 1). With H2-enhanced mixotrophy (Fig. 5a), product yields based on sugar mass increased 49–100% for all metabolites, aside from acetate, and the quantities of H2 required for supplementation range from 0.017–0.065 g H2 g glucose /C0 (Fig. 5b). As with the mixotrophic results, the chemicals that require the most reducing energy also require the largest amount of H2 to fix all CO2, and the amount of exogenous H2 required is directly proportional to the NAD(P)H to acetyl-CoA ratio (Table 1). Since H2-enhanced mixotrophy removes electron constraints, the chemicals that showed the smallest yield improvements from heterotrophy to mixotrophy show the largest yield improvements from mixotrophy to H2-enhanced mixotrophy. For syngas-enhanced mixotrophy, similar yield improvements can be achieved using CO as the electron donor though CO2 is generated in the process. In order to minimize net CO2 production, a mixture of CO and H2 is needed, and the ideal mixture is dependent upon the target metabolite. In this case, yields can be increased beyond those calculated here because the gaseous carbon can be fixed into metabolites, as seen in Fig. 4a,c.

**Discussion**

Mixotrophic behaviour has been previously reported, such as the stoichiometric conversion of sugar into acetate by MTA25 and the co-consumption of sugar and C1 feedstocks for A. woodii12 and E. limosum14. However, the potential of mixotrophy to increase product yields beyond previous theoretical limits, and its application to industrial biotechnology has largely been overlooked. Here we more thoroughly demonstrate that mixotrophy takes place and is a general trait of all four acetogens tested. Moreover, we genetically engineered a CLJ strain to produce acetone at a product yield that is 138% of the previous theoretical maximum, which was calculated based on heterotrophy alone. This significant increase in product yield could convert a previously uneconomical fermentation process into an economically viable one.

Though mixotrophy is a general trait among acetogens, allowing for the application of different strains for different metabolites or processes, the work in this study largely used CLJ as a mixotrophic host for several reasons. First, CLJ has proven to be genetically malleable, with several key genetic tools now available, including plasmid overexpression15,16, inducible plasmid expression21, chromosomal deletions16,26 and chromosomal integrations27. The ability to genetically manipulate CLJ allows for the possibility to increase production of minor metabolites or introduce pathways to produce non-native metabolites, like acetone in this study. Another trait that makes CLJ a desirable mixotrophic host is the surprising result from the 13C-labelling analysis that in the presence of both sugar and gases, CLJ produced more acetate from the gaseous substrate than the sugar substrate. Indeed, over 70% of the produced acetate was
only fructose with \( \text{H}_2 \) and fructose with a syrupa mixture (\( \text{CO}_2 : \text{CO}_2 : \text{H}_2 : \text{N}_2, 55:20:10:15 \)), however many different organic and inorganic substrates can be used such as other sugars, gas compositions, methanol, formate, and other C1 substrates. Gaining a better understanding of the effect of all these possible substrates and their impact on product formation is crucial to best pair potential feedstocks for producing products of interest. An example of this effect can be observed in the syngas-enhanced mixotrophy results (Fig. 4). In those experiments, CLJ and CAU outperformed MTA and ELM in terms of total product yield from sugar (195 and 165% yields versus 119 and 86% yields, Fig. 4a).

However, it is unclear whether these differences in performance are related to the strains themselves or rather the conditions tested. For example, the syngas composition may not have been ideal for MTA or ELM.

Beyond a fundamental analysis, the data from the high cell density continuous fermentation (Fig. 3) constitute a foundation for further development to achieve industrially relevant productivities, unprecedented mass yields and increased product titers.

### Methods

#### Strains and growth conditions

C. *ljungdahlii* DSM-13528 (CLJ), *C. autoethyogenes* DSM-10061 (CAU), *E. limosum* DSMZ-20543 (ELM) and *M. thermoautoctica* DSM-521 (MTA) were obtained from DSMZ (Braunschweig, Germany). All cultures were grown in sealed serum bottles in a shaking incubator (150 r.p.m.) at 37°C, except for MTA which was incubated at 55°C. They were cultured anaerobically in American Type Culture Collection (ATCC) medium 1754 with 10 g l\(^{-1}\) of fructose, with the exception of ELM which was grown in a modified ATCC medium 1754 (supplemented with 10 g l\(^{-1}\) MES, pH 6.0) with 5 g l\(^{-1}\) of fructose. Growth was monitored by measuring the optical density at 600 nm (OD\(_{600}\)). A 5% inoculum of mid-exponential phase (OD\(_{600}\) of 0.8–1.5) was used to inoculate 160 ml serum bottles (Wheaton), with 50 ml of culture media and 110 ml of gas headspace. For autotrophic and syngas enhanced mixotrophic cultures, the headspace was pressurized to 30 psig with syngas (\( \text{CO}_2 : \text{H}_2 : \text{N}_2, 55:10:20:15 \)), except for ELM which was pressurized to 20 psig. MTA was grown in a mixture without CO (\( \text{CO}_2 : \text{H}_2 : \text{N}_2, 80:20)\). Heterotrophic and mixotrophic cultures were pressurized to 20 psig with \( \text{N}_2 \). The pH of the cultures was monitored and kept between 5.0–6.5 by adding 4M \( \text{NH}_4\text{OH} \).

#### 13C Metabolite labelling

CLJ and CAU were grown as described above with 10 g l\(^{-1}\) of 13C-fructose. The syngas-enhanced mixotrophic and autotrophic cultures contained a mixture of syngas (\( \text{CO}_2 : \text{H}_2 : \text{N}_2, 55:10:20:15 \)) with all carbons labelled. Samples were taken at early, mid and late exponential phase and metabolites and biomass was analysed for 13C content. Gas chromatography–mass spectrometry analysis of 13C labelling was performed on an Agilent 7890A GC system equipped with a DB-5 MS capillary column connected to a Waters Quattro Micro Tetand Mass Spectrometer operating under ionization by electron impact at 70 eV (ref. 29).

#### Construction of CLJ ASADH (ptCTA)

The SADH gene in CLJ (CLJU_c24860) was deleted from the genome, as this enzyme readily converts acetone into isopropanol22. A replicating plasmid was constructed and transformed into CLJ to completely delete the gene using a homologous recombination technique. Two regions of homology, each about 1,400 bp in length, were PCR amplified from CLJ genomic DNA using the primers listed in Supplementary Table 3. These two regions were then PCR assembled, with a NotI restriction enzyme site in-between the two regions, and ligated into a PCB862GW/TOPO TA entry plasmid (ThermoFisher Scientific). The chloramphenicol and thiamphenicol resistance gene from pSOS95-Cm was then PCR amplified, digested with NotI and ligated into the two regions of homology, each about 1,400 bp in length, were PCR amplified from CLJ genomic DNA using the primers listed in Supplementary Table 3. These two regions were then PCR assembled, with a NotI restriction enzyme site in-between the two regions, and ligated into a PCB862GW/TOPO TA entry plasmid (ThermoFisher Scientific). The custom deletion plasmid was then transformed into the genome of CLJ. The SADH gene in CLJ (CLJU_c24860) was deleted from the genome, as this enzyme readily converts acetone into isopropanol22. A replicating plasmid was constructed and transformed into CLJ to completely delete the gene using a homologous recombination technique. Two regions of homology, each about 1,400 bp in length, were PCR amplified from CLJ genomic DNA using the primers listed in Supplementary Table 3. These two regions were then PCR assembled, with a NotI restriction enzyme site in-between the two regions, and ligated into a PCB862GW/TOPO TA entry plasmid (ThermoFisher Scientific). The custom deletion plasmid was then transformed into the genome of CLJ. The SADH gene in CLJ (CLJU_c24860) was deleted from the genome, as this enzyme readily converts acetone into isopropanol22. A replicating plasmid was constructed and transformed into CLJ to completely delete the gene using a homologous recombination technique. Two regions of homology, each about 1,400 bp in length, were PCR amplified from CLJ genomic DNA using the primers listed in Supplementary Table 3. These two regions were then PCR assembled, with a NotI restriction enzyme site in-between the two regions, and ligated into a PCB862GW/TOPO TA entry plasmid (ThermoFisher Scientific). The custom deletion plasmid was then transformed into the genome of CLJ.

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**Table 1 | Correlation between NAD(P)H:acetyl-CoA ratio in improvement in product yields with mixotrophy.**

| NAD(P)H: acetyl-CoA ratio | Increase in yield of mixotrophy over heterotrophy |
|---------------------------|-----------------------------------------------|
| 0                          | 53%                                           |
| 0.5                       | 36%                                           |
| 1.0                       | 22%                                           |
| 1.5                       | 11%                                           |
| 2                         | 2%                                            |
| 3                          | 0%                                            |
CoA-transferase gene from CAC (CA_P0163&0164, 8 mixture. Batch fermentations of CLJ erythromycin. Colonies which grew on thiamphenicol but were sensitive to ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms12800

Fructose concentration in the feed was increased to as high as 80 g l\(^{-1}\) during the course of building cell density, and the concentration of clarithromycin was

Batch fermentations of CLJ ASADH (pCTCA). Cultures of CLJ ASADH (pCTCA) were carried out in anaerobic ATCC medium 1754 with 5 g l\(^{-1}\) fructose and 100 g ml\(^{-1}\) erythromycin in sealed 160-ml serum bottles, in a similar manner as described above. The atmosphere was either 30 psig N\(_2\) or 30 psig of an H\(_2\)/N\(_2\) mixture.

High density cell recycle fermentations. A 10% inoculum of mid-exponential phase (OD\(_{600}\) 0.8–1.5) CLJ ASADH (pCTCA) culture was used to inoculate a 31 bioreactor (Appikon) with a 1:91 working volume of anaerobic ATCC medium 1754 with 5 g l\(^{-1}\) fructose and 5 ml g\(^{-1}\) of clarithromycin. Cultures were sparged with N\(_2\) for the first 24 h to maintain an anaerobic environment as the cultures entered exponential growth. Once the cultures reached an OD\(_{600}\) of 1.5, cell-recycle was initiated. Fermentation pH was controlled from dropping below 5.0 using 4M NH\(_4\)OH. Fermenter feeding and permeate rates were balanced to allow for optimal cell growth and production. Once the cell density reached an OD\(_{600}\) of 35–60, at which time a harvest was started that was equal to the critical dilution rate that maintained a constant cell density in the fermentor. Fructose concentration in the feed was increased to as high as 80 g l\(^{-1}\) during the course of building cell density, and the concentration of clarithromycin was maintained at 5 mg l\(^{-1}\).

Detection of metabolites. Culture supernatant samples were quantified using an Agilent HPLC instrument with a Bio-Rad Aminex HPX 87H column using a 5 mM H\(_2\)SO\(_4\) mobile phase. Gas samples were analysed using an Agilent GC instrument with a Supelco 60/80 Carboxen-1,000 column, using according to the manufacturer’s recommendations.

RNA isolation and quantitative reverse transcription qRT-PCR. RNA was extracted using the RNeasy Kit (Qiagen) with a modified protocol as follows: cells were thawed on ice and washed and resuspended in 220 ml RNase-free SET buffer + 20 ml proteinase K. Samples were sonicated for 10 min using 15 s pulse on/off at 40% amplitude in a Fisher Scientific Sonic Dismembrator. In all, 1 ml of Trizol was added to each sample, split in two and another 400 ml Trizol was added to have the final volume of ~1 ml. 200 ml of ice-cold chloroform were added to the sample and mixed for 15 s, then incubated at room temperature for 3 min. Samples were spun at ~12,000g for 15 min at 4\(^\circ\)C. Upper aqueous phase was mixed with 500 ml of 25 vol% ethanol and RNase-free proteinase K was added per Incubated overnight at 50\(^\circ\)C. The resulting concentration was determined using a NanoDrop (Thermo Scientific). Concentrations ranged from ~50 ng ml\(^{-1}\) to ~600 ng ml\(^{-1}\) depending on the sample. Complementary DNA (cDNA) was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems). In all, 1 ml of cDNA was used in qRT-PCR experiments. All qRT-PCR experiments were performed in triplicate for 15 s pulse on/off at 50% amplitude and monitored for 15 s pulse on/off at 50% amplitude and monitored for 380–395 (2012).

Data availability. All relevant data are included with the manuscript (as figure source data or supplementary information files), and all data is available upon reasonable request from the corresponding author.

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Author contributions

S.W.J. performed many of the fermentation experiments, analysed the data and wrote the paper. A.G.F. performed some of the fermentation experiments, the qRT–PCR, western blots and wrote the paper. J.A. and M.R.A. performed the 13C analysis. E.T.P. analysed data and wrote the paper. B.P.T. designed the study, analysed the data and wrote the paper. S.W.J. A.G.F. and E.D.C. contributed equally to the study. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: White Dog Labs is commercializing a mixotrophy-based fermentation process and has filed a patent application PCT:US2016:019760.

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