

Hexanol biosynthesis from syngas by *Clostridium carboxidivorans* P7 is enhanced by in-line extraction with a biocompatible solvent to avoid product toxicity

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Research

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Abstract

Background *Clostridium carboxidivorans* P7 has the rare ability to metabolize syngas – a mixture of H₂, CO and CO₂ – by converting it directly into industrially relevant alcohols (hexanol, butanol and ethanol) and the corresponding acids (caproate, butyrate and acetate). The product titers and ratios are highly dependent on the fermentation parameters and the composition of the syngas and growth medium. The hexanol titers produced by *C. carboxidivorans* P7 have recently been improved by optimizing these conditions, but little is known about the toxicity of hexanol towards *Clostridium* species. We hypothesized that the hexanol titers currently produced by *C. carboxidivorans* P7 are limited by product toxicity.

Results We tested our hypothesis by exposing *C. carboxidivorans* P7 to different concentrations of hexanol and found that growth inhibition started at 10–12 mM, with an IC₅₀ of 17.5 ± 1.6 mM. The presence of 20 mM hexanol was acutely toxic to *C. carboxidivorans* P7 as well as the model acetogen *C. ljungdahlii*, which does not produce hexanol. To avoid product toxicity, we added a biocompatible solvent (oleyl alcohol) to fed-batch bottle fermentations of *C. carboxidivorans*. This increased the total hexanol titers by 2.5-fold from 9.4 to 24.4 mM. Cell growth and product profiles in the aqueous phase after fermentation were similar in cultures with and without oleyl alcohol. The extraction phase contained high levels of hexanol (436 ± 101 mM) and butanol (100.3 ± 17.8 mM) as well as low levels of ethanol and traces of caproate.

Conclusions Product toxicity was confirmed as a limiting factor during the conversion of syngas to hexanol by *C. carboxidivorans* P7. The addition of oleyl alcohol as a biocompatible solvent led to a significant increase in hexanol titers by facilitating the efficient and selective extraction of this product. We have therefore identified an in-line extraction strategy that increases the yield of hexanol and should allow for further improvements by genetic adaptation and/or process optimization.

Background

The utilization of industrial waste gases to produce fuels and platform chemicals could help to counter the effects of climate change. The production of medium-chain alcohols such as butanol and hexanol is particularly attractive due to their higher energy density and lower hygroscopic activity compared to ethanol [1]. These alcohols can be used directly as so-called drop-in fuels because they are compatible with current combustion engine designs, and they can also be used as industrial solvents or platform chemicals for conversion into polymers [2]. Recent publications by companies such as Evonik and Siemens concerning butanol and hexanol production from syngas are evidence for increasing interest in this process from an industrial perspective [3].

One of the most abundant industrial waste gases is synthesis gas (syngas), a mixture of H₂, CO and CO₂. Syngas can be metabolized by a heterogeneous group of bacteria known as acetogens. Acetogenesis is a

physiological rather than a phylogenetic trait and is therefore distributed across 23 genera, with many representatives in the genus *Clostridium* [4]. Unlike chemical catalysts, these bacteria can metabolize syngas under ambient conditions and can accommodate syngas substrates varying in composition and impurity levels [5, 6]. Sustainable sources of syngas include off-gases from steel mills and incineration plants, so the conversion of such resources can help to offset industrial greenhouse gas emissions. The acetogenic strain *Clostridium carboxidivorans* P7 was isolated by screening for species that grow on CO [7] and is noted for its rare ability to produce C6 compounds like hexanol directly from syngas [8–10]. Fermentation with *Clostridium* species usually involves two production phases. The first is an exponential growth phase that mainly involves acetogenesis and is directly linked to energy conservation [11–13]. When the accumulation of acetate and other primary products begins to inhibit growth, the bacteria shift from acetogenesis to solventogenesis. The primary acidic products are taken up from the medium and reduced further to the corresponding alcohols. This allows the cells to conserve more energy and is often correlated with sporulation, although sporulation and solventogenesis tend to be regulated independently in *Clostridium* species [14–18].

Acetogens metabolize syngas via the Wood–Ljungdahl pathway, also known as the reductive acetyl-CoA pathway. Two molecules of C1 substrate are reduced to a coenzyme A-bound acetyl group using electrons derived from H₂ or CO. Acetyl-CoA can then be converted to the C2 products acetate and ethanol for energy conversion, or utilized to produce biomass [12]. Some acetogens, including *C. carboxidivorans* P7, can extend acetyl-CoA to synthesize the C4 products butyrate and butanol, and the C6 products caproate and hexanol [7, 10]. The ability of *C. carboxidivorans* P7 to produce caproate and hexanol from syngas can be enhanced by medium and process optimization [10, 19, 20]. For example, the optimization of trace element composition significantly improved bacterial growth, leading to yields of 1.33 g/L (13.3 mM) hexanol [19]. Furthermore, a two-step fermentation process has been developed to overcome the limitations of biphasic growth, where most cell growth is restricted to acetogenesis and growth inhibition triggered by the switch to solventogenesis as the medium becomes more acidic. The first fermenter is held at pH 6 for acetogenesis, allowing the accumulation of biomass, acetate and ethanol, then the medium is renewed and some of the broth is flushed to a second fermenter with cell retention at pH 5 for solventogenesis. This allows longer fermentation runs because the first fermenter continuously provides cells and substrate for chain elongation [20].

Although the effect of acids during fermentation is well understood, there are no data concerning the potential toxicity of hexanol towards *Clostridium* species, including *C. carboxidivorans* P7 [9]. IC₅₀ values for ethanol (35 g/L) and butanol (14.5 g/L) have been determined for *C. carboxidivorans* P7 grown with CO as a sole carbon and energy source [21]. In another study, the reported IC₅₀ value of butanol for *C. carboxidivorans* P7 was 4.12 g/L, with complete inhibition at 13.92 g/L [22]. Given that alcohol toxicity increases with chain length and the corresponding nonpolar characteristics of the carbon chain, the IC₅₀ of hexanol in *Clostridium* species is expected to be even lower than the values reported for butanol and ethanol, as already shown in *Escherichia coli* [23].

Product inhibition during alcohol production can be avoided by the use of oleyl alcohol (octadecenol) as a biocompatible extraction solvent, as already reported for acetone–butanol–ethanol (ABE) fermentation [24, 25]. Hydrophobic extraction agents are advantageous because the product is transferred to an organic phase, reducing the concentration of toxic products in the aqueous fermentation broth containing the cells. Furthermore, the low polarity and low solubility of hexanol can be exploited to concentrate the product in the extraction phase. Phase separation also facilitates continuous product harvesting because, without constant agitation, the lipophilic phase containing the hexanol forms a layer on top of the fermentation broth. The extraction solvent is therefore easy to remove and replace, providing an opportunity for extended fermentation runs with minimal downtime. Finally, the large difference between the boiling points of hexanol (157 °C) and oleyl alcohol (330–360 °C) allows rapid product recovery by distillation and potential recycling of the solvent. We therefore measured the toxicity of hexanol towards *C. carboxidivorans* P7 and the model acetogen *C. ljungdahlii*, and evaluated the use of oleyl alcohol as an in-line extraction solvent during the production of hexanol from syngas in order to increase hexanol titers.

Results

The acute toxicity of hexanol

The acute toxicity of hexanol was established by conducting growth inhibition assays. Cells growing in anaerobic glass roll tubes containing modified minimal medium with syngas as a growth substrate were exposed to 0, 10, 20, 30 or 40 mM hexanol during the exponential growth phase (**Figure 1A,B**). Growth was measured by turbidimetry, and corresponding OD₆₀₀ values were calculated from standard curves so that growth behavior could be compared in roll tubes and serum bottles. To determine whether the ability to produce hexanol confers resistance to this product, we compared the growth of the natural hexanol producer *C. carboxidivorans* P7 with that of the model acetogen *C. ljungdahlii*, which does not produce hexanol. Interestingly, despite the difference in metabolic capability, we observed no difference in hexanol tolerance between these strains. There was no effect on the growth of either strain in the presence of 10 mM hexanol, but exposure to ≥ 20 mM caused the turbidity to increase immediately after the hexanol was added and to remain stable thereafter. Furthermore, gas consumption ceased in cultures with hexanol concentrations ≥ 20 mM. This indicated the significant inhibition of growth and syngas utilization. To determine whether the cells were killed or merely dormant, 500- μ L aliquots of cells were removed after 24 h and transferred to fresh medium without hexanol to see whether recovery was possible. The cultures originally exposed to 0 and 10 mM hexanol recovered fully, whereas only 50% of the cultures originally exposed to 20 mM hexanol were able to continue growing. There was no regrowth in the cultures exposed to higher concentrations of hexanol. These findings indicate that exposure to 20 mM hexanol for 24 h killed nearly all of the cells, and a 500- μ L aliquot contained on average less than one viable cell. At higher hexanol concentrations, significant macroscopic flocculation of the cells was observed (**Figure 1C**), indicating that hexanol has a negative effect on the cell membranes. Flocculation was also observed during fed-batch bottle fermentations after 3–4 days, and has been reported in other studies for the production of alcohols [19].

Hexanol titers at the onset of inhibition and calculation of the IC₅₀

Having evaluated the acute toxicity of hexanol, we next determined the minimal inhibitory concentration and IC₅₀ for *C. carboxidivorans* P7. As above, cells were grown in anaerobic glass tubes containing modified minimal medium and syngas as a growth substrate, but this time the medium was supplemented with 0, 12, 14, 15, 16, 18, 20 or 22 mM hexanol before inoculation (**Figure 2**). Exponential growth began immediately after inoculation. On the second day of growth in the presence of hexanol, the growth rates decreased rapidly and a near linear growth profile was observed instead of exponential growth. Both the initial growth rates and final biomass yields were lower in cultures with higher hexanol concentrations. Cultures containing 22 mM hexanol did not show significant growth, and cultures containing 20 mM hexanol doubled once on the first day and then stagnated. After two days of growth, cultures containing 12 mM hexanol achieved only ~50% of the biomass yield of the control, confirming that significant growth inhibition occurred even at low hexanol titers.

The IC₅₀ of hexanol was 17.5 ± 1.6 mM based on normalized initial growth rates. Given the immediate effect of hexanol on turbidity (**Figure 1**), the apparent growth rates of cultures containing higher titers of hexanol are likely to be greater than the real growth rates. The immediate effect of hexanol on turbidity appeared to be dependent on both cell density and hexanol concentration, so it was not possible to calculate adjusted growth rates from the empirical data. The real IC₅₀ is therefore likely to be slightly lower than the calculated value (approximately 15–17 mM).

Oleyl alcohol avoids product toxicity

Oleyl alcohol has been widely used as an extraction agent in ABE fermentation but has not been tested with gaseous substrates. We therefore cultivated cells in modified minimal medium with syngas as above, but also added 5% (v/v) oleyl alcohol and 100 mM hexanol. Both *C. carboxidivorans* P7 and *C. ljungdahlii* were able to grow robustly, confirming that oleyl alcohol does not impair growth even with gaseous substrates and is able to detoxify the medium with hexanol titers of at least twice the concentration soluble in water. However, the oleyl alcohol formed microscopic bubbles or vesicles that increased the optical density of the medium over time (data not shown), so it was not possible to collect accurate values directly. For subsequent experiments, cells were therefore harvested and washed by centrifugation before OD₆₀₀ values were determined.

In-line hexanol extraction during fed-batch bottle fermentation

To evaluate the effect of the extraction solvent on product formation, fed-batch bottle fermentations were carried out in the presence or absence of 4% (v/v) oleyl alcohol. We inoculated *C. carboxidivorans* P7 cultures into 25 mL medium in 250-mL serum flasks and fed them with syngas (65% CO, 15% CO₂, 15% N₂, 5% H₂) at 1 bar overpressure, with gas-phase renewal every 24 h for 4 days. After 3 further days, the final OD₆₀₀ values were 5.7 ± 0.3 in the control culture and 5.9 ± 0.6 in the culture containing 4% (v/v) oleyl alcohol, confirming our earlier observation that oleyl alcohol does not affect growth. The

experiments were carried out at 30 °C because this supported stable growth and approximately doubled the hexanol titers compared to growth at 37 °C (data not shown). Cells in the exponential growth phase produced mostly acetate and ethanol, and later butyrate and butanol. Hexanol production started during the late exponential to early stationary phase, and the acetate and ethanol titers decreased (**Figure 3**). The final hexanol titers in the aqueous phase after 7 days were 9.4 ± 2.5 mM in the control culture and 7.0 ± 0.6 in the culture containing 4% oleyl alcohol, whereas the titers of all other fermentation products were similar in both cultures (**Figure 4A**). In the oleyl alcohol phase, the hexanol concentration was 436 ± 101 mM (**Figure 4B**), corresponding to 17.4 mM for the entire culture volume. When added to the 7.0 mM hexanol in the aqueous phase, the overall hexanol titer was 24.4 mM, representing a 2.5-fold increase compared to the control without oleyl alcohol. Two thirds of the total hexanol was found in the oleyl alcohol phase, corresponding to a concentration factor of 60 over the aqueous phase. In addition to hexanol, the oleyl alcohol phase contained 100.3 ± 17.8 mM butanol (concentration factor = 5.4) and traces of caproate (**Figure 5**). These findings not only confirm that oleyl alcohol is an efficient hexanol extraction solvent during the fermentation of syngas, but also shows its positive effect on hexanol titers by removing the toxic product from the fermentation broth.

Discussion

Clostridium carboxidivorans P7 has the unusual ability to produce hexanol directly from syngas, but the toxicity of hexanol has not been determined in *Clostridium* species and current maximum titers may be limited by product toxicity. Accordingly, we tested the acute toxicity of hexanol in *C. carboxidivorans* P7 and *C. ljungdahlii* (which does not produce hexanol) and found that almost all cells were killed by exposure to 20 mM (2 g/L) hexanol for 24 h. Intuitively, one might expect greater hexanol tolerance in a natural producer than an organism without natural exposure to this compound. However, the similar sensitivity we observed is probably explained by the natural environment of *C. carboxidivorans* P7, which was isolated from an agricultural settling lagoon [7]. This environment is not an optimized medium for hexanol production, so the titers of hexanol would be much lower than we achieved in the laboratory, and in any case the lagoon would allow hexanol to diffuse away from its source, reducing the local concentration. Hexanol production by *C. carboxidivorans* P7 in its natural environment is therefore unlikely to impose selective pressure for improved tolerance.

We also established the minimal inhibitory concentration and calculated an apparent IC_{50} of 17.5 ± 1.6 mM (1.8 g/L) for hexanol in *C. carboxidivorans* P7, although the real IC_{50} may be even lower because hexanol increases the turbidity of the cultures. Growth inhibition was more severe on the second day, with significant growth impairment observed in the presence of 12 mM hexanol. This suggests that the toxicity of hexanol increases with longer exposure times, that toxicity is induced by the accumulation of metabolic products, or there is a combination of both effects. The IC_{50} of hexanol was much lower than the values reported for ethanol (35 g/L, 759.7 mM) and butanol (14.5 g/L, 195.6 mM) [21] although a lower value of 4.12 g/L was reported for butanol in a different study [22]. The higher toxicity of longer-chain alcohols is anticipated because such products are less polar and interact more strongly with lipid

membranes [23]. Hexanol toxicity mainly reflects its effect on membrane fluidity, as demonstrated in *E. coli* [26]. The macroscopic flocculation of *C. carboxidivorans* P7 cells in the presence of higher concentrations of hexanol indicates a similar mode of toxicity in *Clostridium* spp. A different mode of toxicity has been demonstrated in the yeast *Saccharomyces cerevisiae*. Strains with enhanced n-hexanol tolerance were shown to carry mutations in the eIF2 and eIF2B complexes that prevented the negative effects of n-hexanol on the translation initiation complex, allowing significantly improved growth in the presence of 0.15% n-hexanol compared to the wild-type strain [27]. We found that both *C. carboxidivorans* P7 and *C. ljungdahlii* were less sensitive to hexanol than *E. coli*, the latter showing 45% inhibition of exponential growth in the presence of 0.625 g/L (6.25 mM) hexanol [23] whereas the inhibition of *C. carboxidivorans* P7 growth in this study started at approximately double this concentration (10–12 mM). The IC₅₀ of hexanol reported for an enrichment culture of methanogens was 1.5 g/L [28], which is similar to the value found in this study.

Extraction with oleyl alcohol showed promising detoxification results and increased the final hexanol titers by 2.5-fold. Our results therefore indicate a strong correlation between the inhibitory hexanol concentration of 10–12 mM and the highest hexanol titers of 13.3 mM produced by *C. carboxidivorans* P7 thus far [19]. Biocompatible extraction solvents such as oleyl alcohol remove toxic products from the fermentation broth and thus achieve higher overall titers. In this study, we produced final titers of 24.4 mM hexanol, an 84% increase over the best previous results [19]. With product toxicity addressed, other factors limiting hexanol production can be investigated, such as substrate availability. One of the most important factors affecting hexanol production is the availability of short-chain substrates for elongation because acetate and ethanol production stops once longer-chain alcohols are synthesized. The recently reported two-step fermentation process provides a constant influx of new substrate by holding the first fermenter at a higher pH for acetogenesis and the second at a lower pH with cell retention for chain elongation and alcohol production [20]. The combination of two-step fermentation with in-line extraction in the second fermenter could therefore increase hexanol titers and running times even further, especially if the extraction solvent were replenished to allow for continuous hexanol extraction.

Conclusions

Product toxicity was confirmed as an important factor limiting hexanol production during the fermentation of syngas by *C. carboxidivorans* P7. The addition of oleyl alcohol as a biocompatible solvent led to a significant increase in hexanol production and facilitated efficient and selective product extraction. Having addressed the challenge of growth inhibition by low titers of the product hexanol, it should now be possible to increase titers far beyond current maximum levels by further strain development and the optimization of process parameters.

Methods

Chemicals, strains and cell cultivation

All chemicals were supplied by Sigma-Aldrich Chemie (Merck, Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany). All gases were supplied by Westfalen (Münster, Germany). Type strains of *C. carboxidivorans* P7 (DSM 15243) and *C. ljungdahlii* (DSM 13528) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Both strains were grown in modified Protein Expression Technology Center (PETC) 1754 medium (ATCC, Manassas, VA, USA) buffered at pH 6 with 100 mM BisTris. The modified minimal medium contained 1 g/L ammonium chloride, 0.1 g/L potassium chloride, 0.2 g/L magnesium sulfate heptahydrate, 0.8 g/L sodium chloride, 0.1 g/L potassium dihydrogen phosphate, 20 mg/L calcium chloride dehydrate and 1 g/L yeast extract. Trace elements and vitamins were prepared as described by ATCC, and 0.5 mg/L resazurin was used as a redox indicator to ensure strictly anaerobic conditions in the medium. Batches of medium were autoclaved prior to the addition of vitamins and trace elements from sterile stock solutions and were left overnight in a Whitley A55 Anaerobic Workstation (Don Whitley Scientific, Herzlake, Germany) in an oxygen-free atmosphere comprising 5% H₂, 10% CO₂ and 85% N₂. Lcysteine (0.75 g/L) was added as a reducing agent. An optimized trace element composition for alcohol production was used for hexanol production experiments [19].

Determination of hexanol toxicity

Pre-cultures of *C. carboxidivorans* P7 and *C. ljungdahlii* were adapted for several generations to grow on modified minimal medium with optimized trace elements [19] and syngas as the growth substrate. Adapted cells were used to inoculate 5-mL cultures in 20-mL anaerobic glass tubes (Glasgerätebau Ochs, Bovenden/Lenglern, Germany) with rubber stoppers, and the headspace was filled with syngas (33.3% CO₂, 33.3% CO and 33.3% H₂). The cultures were maintained at 37 °C with constant agitation at 150 rpm. Cell growth was measured using an HI93703 microprocessor turbidity meter (Hanna Instruments Deutschland, Vöhringen, Germany) and the OD₆₀₀ was calculated using a calibration curve. Once growth was observed, hexanol was added from a pure, sterile, anaerobic stock solution via a 10- μ L SGE syringe (Trajan Scientific and Medical, Ringwood, Australia). Hexanol extraction with technical grade 85% oleyl alcohol (Merck) was tested under the same experimental conditions as described above, but the medium was supplemented with 100 mM hexanol and 5% (v/v) oleyl alcohol from sterile, anaerobic stock solutions.

Determination of minimal inhibitory concentrations and IC₅₀

The onset of growth inhibition was investigated using the same cultivation setup as described above, but the syngas composition was adjusted (65% CO, 15% N₂, 15% CO₂ and 5% H₂) and the gas phase was renewed daily to avoid substrate limitation. Cell growth was monitored by turbidimetry and OD₆₀₀ values were calculated using a calibration curve as above. Malthusian growth during the exponential growth phase was calculated using GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA). The experimental setup allowed only three measurements to be taken during this time period, so the experiment was carried out five times (independent biological replicates) with three technical replicates per experiment. Growth rates were normalized against the control without hexanol and were plotted as

percentages against hexanol concentrations. Four-factor sigmoidal dose–response curves and IC_{50} values were calculated using the same program.

Fed-batch bottle fermentation

Adapted cell cultures were inoculated into 25 mL modified minimal medium with optimized trace elements [19] in 250-mL serum bottles and were fed with syngas (65% CO, 15% N₂, 15% CO₂ and 5% H₂) at 1 bar overpressure. The cells were incubated at 30 °C while shaking at 150 rpm. Growth was determined by measuring the OD₆₀₀ after washing the cells by centrifugation for 1 min at 13,000 × g and resuspending them in water. The gas phase was renewed after 1, 2, 3 and 4 days. For the extraction experiments, we added 4% (v/v) oleyl alcohol as above. Samples were drawn via a sterile syringe and stored at – 20 °C for subsequent analysis. At the end of the experiment, the oleyl alcohol and fermentation broth were separated by centrifugation and stored in separate tubes at – 20 °C for analysis by gas chromatography.

Determination of product concentrations

Concentrations of fermentation products in the aqueous phase were measured by gas chromatography as previously described [29]. Samples in oleyl alcohol were diluted 1:100 in pure methanol and then handled mostly in the same manner as the aqueous samples, although the final hold time during gas chromatography was increased to 12 min.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SJ, GP and PK designed the experiments. The experiments were conducted by PK and the data were analyzed by all authors. GP and SJ conceived the project. The manuscript was written by PK and all authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Ethical approval and consent to participate

Not applicable.

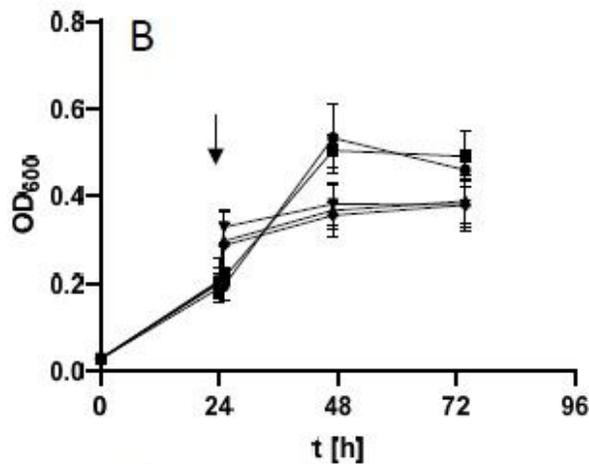
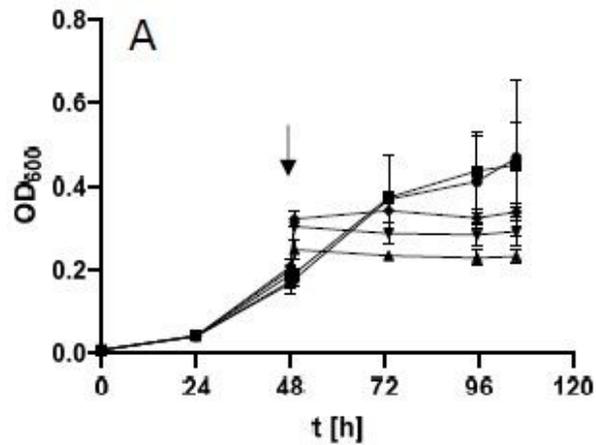
References

1. Köpke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Dürre P: ***Clostridium ljungdahlii* represents a microbial production platform based on syngas.** *Proc Natl Acad Sci U S A* 2010, **107**(29):13087-13092.
2. Dürre P: **Biobutanol: an attractive biofuel.** *Biotechnol J* 2007, **2**(12):1525-1534.
3. Haas T, Krause R, Weber R, Demler M, Schmid G: **Technical photosynthesis involving CO₂ electrolysis and fermentation.** *Nature Catalysis* 2018, **1**(1):32-39.
4. Drake HL, Gossner AS, Daniel SL: **Old acetogens, new light.** *Ann N Y Acad Sci* 2008, **1125**:100-128.
5. Daniell J, Köpke M, Simpson SD: **Commercial Biomass Syngas Fermentation.** *Energies* 2012, **5**(12):5372-5417.

6. Griffin DW, Schultz, M. A.: **Fuel and Chemical Products from Biomass Syngas: A Comparison of Gas Fermentation to Thermochemical Conversion Routes.** *Wiley Online Library* 2012.
7. Liou JS, Balkwill DL, Drake GR, Tanner RS: ***Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov.** *Int J Syst Evol Microbiol* 2005, **55**(Pt 5):2085-2091.
8. Fernandez-Naveira A, Veiga MC, Kennes C: **Selective anaerobic fermentation of syngas into either C2-C6 organic acids or ethanol and higher alcohols.** *Bioresour Technol* 2019, **280**:387-395.
9. Fernandez-Naveira A, Abubackar HN, Veiga MC, Kennes C: **Production of chemicals from C1 gases (CO, CO₂) by *Clostridium carboxidivorans*.** *World J Microbiol Biotechnol* 2017, **33**(3):43.
10. Phillips JR, Atiyeh HK, Tanner RS, Torres JR, Saxena J, Wilkins MR, Huhnke RL: **Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: Medium development and culture techniques.** *Bioresour Technol* 2015, **190**:114-121.
11. Schuchmann K, Müller V: **Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria.** *Nat Rev Microbiol* 2014, **12**(12):809-821.
12. Müller V: **Energy conservation in acetogenic bacteria.** *Appl Environ Microbiol* 2003, **69**(11):6345-6353.
13. Bertsch J, Müller V: **Bioenergetic constraints for conversion of syngas to biofuels in acetogenic bacteria.** *Biotechnol Biofuels* 2015, **8**(1):210.
14. Husemann MH, Papoutsakis ET: **Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations.** *Biotechnol Bioeng* 1988, **32**(7):843-852.
15. Dürre P, Hollergschwandner C: **Initiation of endospore formation in *Clostridium acetobutylicum*.** *Anaerobe* 2004, **10**(2):69-74.
16. Jones DT, Woods DR: **Acetone-butanol fermentation revisited.** *Microbiol Rev* 1986, **50**(4):484-524.
17. Long S, Jones DT, Woods DR: **Sporulation of *Clostridium acetobutylicum* P262 in a Defined Medium.** *Appl Environ Microbiol* 1983, **45**(4):1389-1393.
18. Alsaker KV, Paredes C, Papoutsakis ET: **Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*.** *Biotechnol Bioeng* 2010, **105**(6):1131-1147.
19. Shen S, Gu Y, Chai C, Jiang W, Zhuang Y, Wang Y: **Enhanced alcohol titre and ratio in carbon monoxide-rich off-gas fermentation of *Clostridium carboxidivorans* through combination of trace metals optimization with variable-temperature cultivation.** *Bioresour Technol* 2017, **239**:236-243.
20. Doll K, Ruckel A, Kampf P, Wende M, Weuster-Botz D: **Two stirred-tank bioreactors in series enable continuous production of alcohols from carbon monoxide with *Clostridium carboxidivorans*.** *Bioprocess Biosyst Eng* 2018, **41**(10):1403-1416.
21. Fernandez-Naveira A, Abubackar HN, Veiga MC, Kennes C: **Carbon monoxide bioconversion to butanol-ethanol by *Clostridium carboxidivorans*: kinetics and toxicity of alcohols.** *Appl Microbiol*

- Biotechnol* 2016, **100**(9):4231-4240.
22. Ramio-Pujol S, Ganigue R, Baneras L, Colprim J: **Effect of ethanol and butanol on autotrophic growth of model homoacetogens.** *FEMS Microbiol Lett* 2018, **365**(10).
 23. Wilbanks B, Trinh CT: **Comprehensive characterization of toxicity of fermentative metabolites on microbial growth.** *Biotechnol Biofuels* 2017, **10**:262.
 24. Roffler SR, Blanch HW, Wilke CR: **In situ extractive fermentation of acetone and butanol.** *Biotechnol Bioeng* 1988, **31**(2):135-143.
 25. Jeon BS, Moon C, Kim BC, Kim H, Um Y, Sang BI: **In situ extractive fermentation for the production of hexanoic acid from galactitol by *Clostridium* sp. BS-1.** *Enzyme Microb Technol* 2013, **53**(3):143-151.
 26. Ingram LO, Vreeland NS: **Differential effects of ethanol and hexanol on the *Escherichia coli* cell envelope.** *J Bacteriol* 1980, **144**(2):481-488.
 27. Davis Lopez SA, Griffith DA, Choi B, Cate JHD, Tullman-Ercek D: **Evolutionary engineering improves tolerance for medium-chain alcohols in *Saccharomyces cerevisiae*.** *Biotechnol Biofuels* 2018, **11**:90.
 28. Blum DJ, Speece R: **A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations.** *Research Journal of the Water Pollution Control Federation* 1991:198-207.
 29. Philipps G, de Vries S, Jennewein S: **Development of a metabolic pathway transfer and genomic integration system for the syngas-fermenting bacterium *Clostridium ljungdahlii*.** *Biotechnol Biofuels* 2019, **12**:112.

Figures



C



Figure 1

Acute hexanol toxicity in *C. ljungdahlii* (A) and *C. carboxidivorans* P7 (B). Cells were grown in anaerobic roll tubes with rubber stoppers on syngas (CO, CO₂ and H₂ in equal parts) in standard minimal medium. After definitive growth was observed at OD₆₀₀ = 0.2, hexanol was added (indicated by the arrow) at concentrations of 0 mM (•), 10 mM (■), 20 mM (▲), 30 mM (▼) and 40 mM (◆), and the gas phase was renewed. Each curve is representative of three independent experiments (biological replicates) each

comprising three technical replicates. (C) Macroscopic effects of 40 mM hexanol on *C. carboxidivorans* P7 after 3 days (right) compared to the control without hexanol (left).

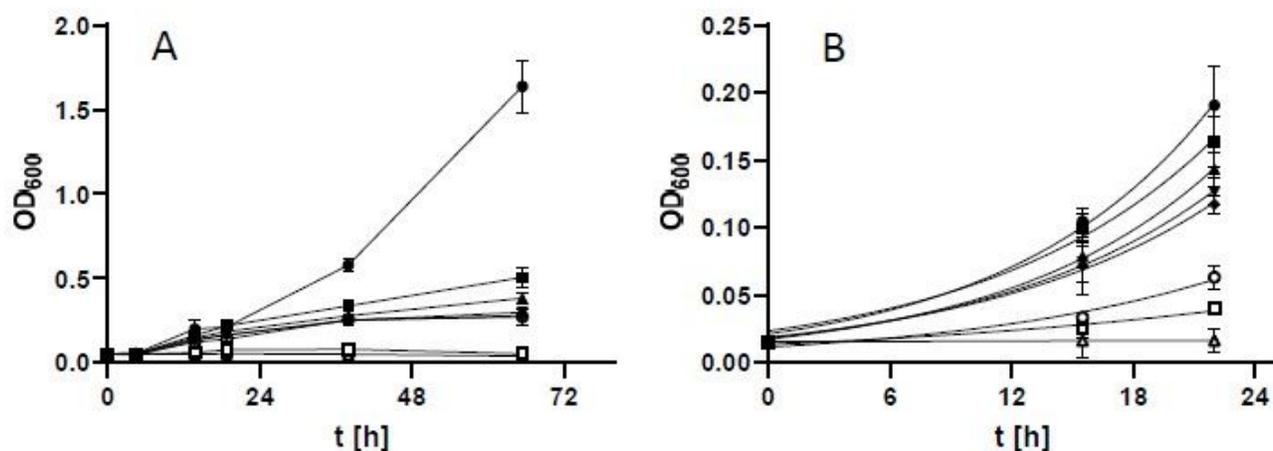


Figure 2

Onset of growth inhibition by hexanol and corresponding IC₅₀ for *C. carboxidivorans* P7. (A) Full growth curves of *C. carboxidivorans* P7 in the presence of 0 mM (●), 12 mM (■), 14 mM (▲), 15 mM (▼), 16 mM (◆), 18 mM (●), 20 mM (□) and 22 mM (△) hexanol. Each curve is representative of three independent experiments (biological replicates) each comprising three technical replicates. (B) Initial exponential growth of *C. carboxidivorans* P7 in the presence of the same hexanol concentrations as above. Each curve is representative of three independent experiments (biological replicates) each comprising three technical replicates.

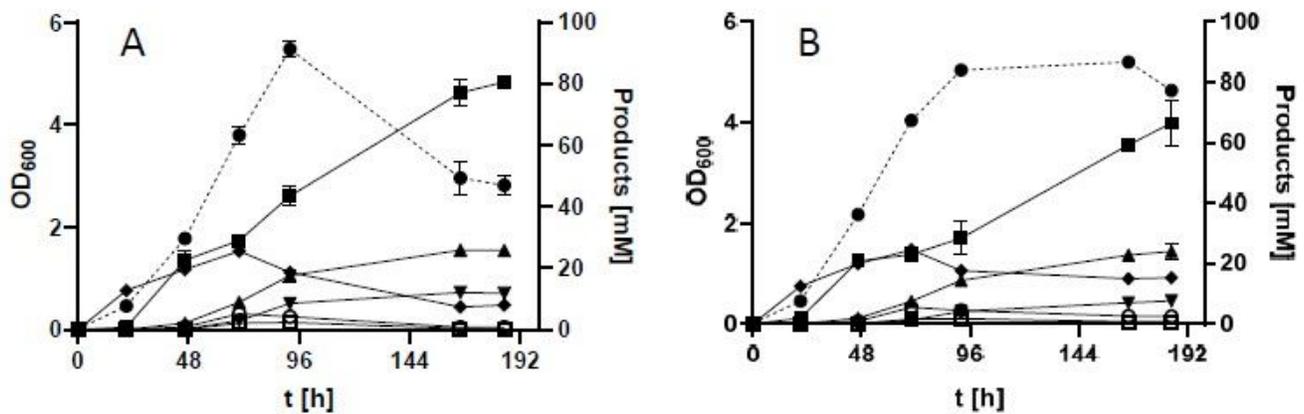


Figure 3

: Growth curves and products in the aqueous phase of *C. carboxidivorans* P7 with in-line extraction. Cells were grown in modified minimal medium (A) and in the presence of 4% (v/v) oleyl alcohol as extraction agent (B). The syngas composition was 65% CO, 15% N₂, 15% CO₂ and 5% H₂. The gas phase was renewed daily for the first 4 days of cultivation. Symbols indicate: \bullet OD_{600} , \blacksquare ethanol, \blacktriangle butanol, \blacktriangledown hexanol, \blacklozenge acetate, \bullet butyrate, \square caproate. Each curve is representative of two independent experiments with technical duplicates.

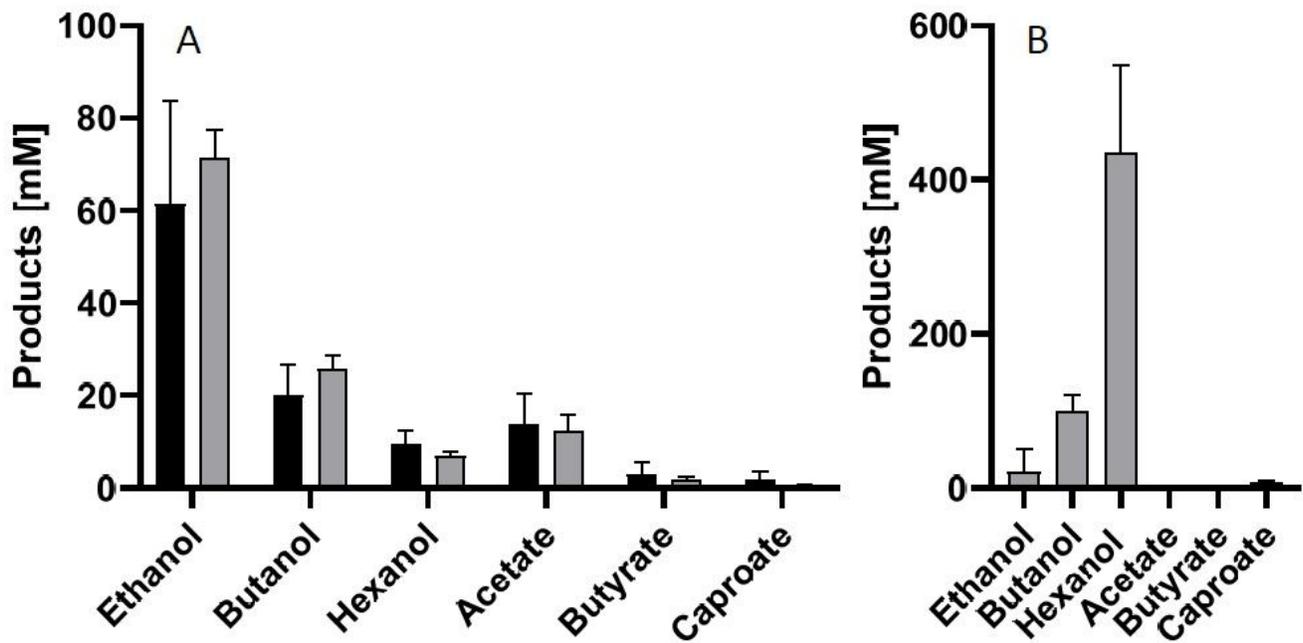


Figure 4

Final products of *C. carboxidivorans* P7 in the presence of 4% oleyl alcohol as an extraction agent. Cells were grown for 7 days in modified minimal medium with syngas as a growth substrate (65% CO, 15% N₂, 15% CO₂ and 5% H₂). The gas phase was renewed daily for the first 4 days of cultivation. (A) Product titers in the aqueous phase with black bars indicating the control culture (no oleyl alcohol) and gray bars indicating cultures grown in the presence of 4% (v/v) oleyl alcohol. (B) Product titers in the extraction phase. Data are means (n=5 replicates from two independent experiments) ± standard deviations.

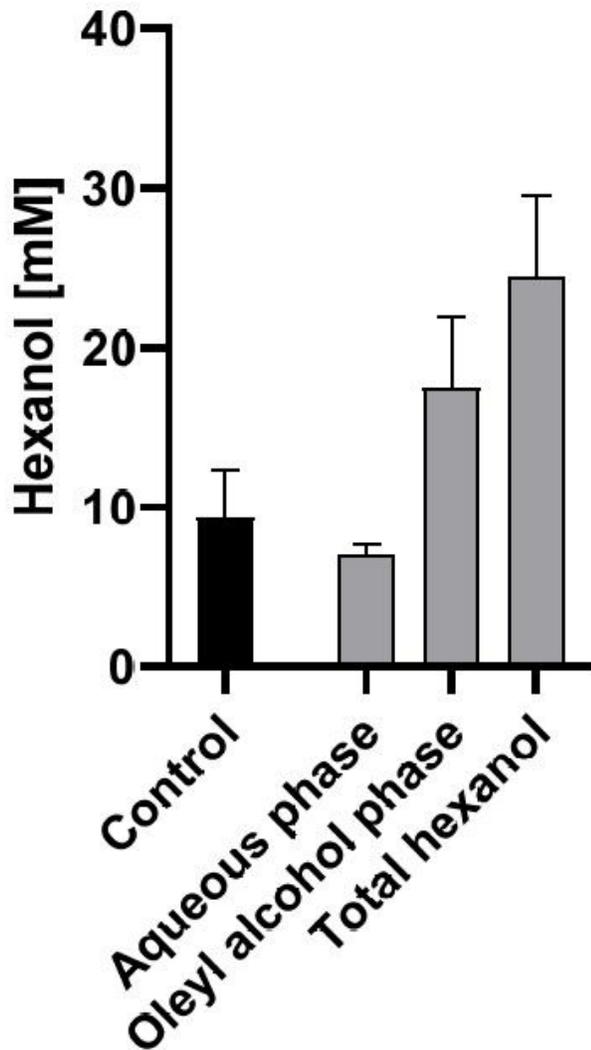


Figure 5

Final hexanol titers of *C. carboxidivorans* P7 in the presence of 4% oleyl alcohol as an extraction agent. Cells were grown for 7 days in modified minimal medium with syngas as a growth substrate (65% CO₂, 15% N₂, 15% CO₂ and 5% H₂). The gas phase was renewed daily for the first 4 days of cultivation. Hexanol titers measured in oleyl alcohol were normalized to the total culture volume for comparison with titers measured in the aqueous phase. Hexanol titers of cultures grown without oleyl alcohol are shown as the negative control. Data are means (n=5 replicates from two independent experiments) ± standard deviations.