



Research article

Hexanol biosynthesis from syngas by *Clostridium carboxidivorans* P7 – product toxicity, temperature dependence and *in situ* extraction

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ABSTRACT

Clostridium carboxidivorans converts syngas into industrial alcohols like hexanol, but titers may be limited by product toxicity. Investigation of IC₅₀ at 30 °C (17.5 mM) and 37 °C (11.8 mM) revealed increased hexanol tolerance at lower temperatures. To avoid product toxicity, oleyl alcohol was added as an extraction solvent, increasing hexanol production nearly 2.5-fold to 23.9 mM (2.4 g/L) at 30 °C. This titer exceeds the concentration that is acutely toxic in the absence of a solvent, confirming the hypothesis that current hexanol production is limited by product toxicity. The solvent however had no positive effect at 37 °C. Furthermore, *C. carboxidivorans* cell membranes adapted to the higher temperature by incorporating more saturated fatty acids, but surprisingly not to hexanol. Corn oil and sunflower seed oil were tested as alternative, inexpensive extraction solvents. Hexanol titers were similar with all solvents, but oleyl alcohol achieved the highest extraction efficiency.

1. Introduction

The utilization of industrial process gases and waste gases to produce fuels and platform chemicals could help counter the effects of climate change. The production of organic alcohols such as butanol is particularly attractive due to their higher energy density and lower hygroscopic activity compared to ethanol (Köpke et al., 2011). Butanol can be used directly as a so-called drop-in fuel because it is compatible with current combustion engine designs (Dürre, 2007), and is also suitable as an industrial solvent or platform chemical for conversion into polymers (Köpke et al., 2011). The similar structure but longer chain length of hexanol makes this alcohol suitable for these and further applications. Recent publications by companies such as Evonik Creavis GmbH and Siemens AG concerning butanol and hexanol production from syngas provide evidence for increasing industrial interest in these processes (Haas et al., 2018).

The traditional route for biotechnological butanol production from renewable resources is acetone-butanol-ethanol (ABE) fermentation. This process has been known for more than a century (Jones and Woods, 1986; Weizmann, 1915) and research is still ongoing (Amiri, 2020; Friedl, 2016; Li et al., 2021; Pudjastuti et al., 2021). In this sugar based fermentation process, biomass from different sources can be used as feedstock. The use of municipal solid waste or lignocellulosic biomass derived from agricultural byproducts allows for the potential reduction of

CO₂ emissions, but requires pretreatment of the substrates as well as downstream processing of the fermentation broth for the isolation of the different end products. The energy needed for these steps significantly influences the return on investment of this process (Amiri, 2020; Friedl, 2016).

Another, more recently applied route for biotechnological alcohol production is syngas fermentation (Dürre, 2016). Since the available products of syngas fermentations are broadly similar to the products of ABE fermentations (mostly short to medium chain organic acids and alcohols), downstream processing technologies like product separation techniques initially developed for ABE fermentation can be adapted for syngas fermentation. Many technologies like adsorption, gas stripping, liquid-liquid extraction, membrane extraction, membrane distillation, reverse osmosis, thermopervaporation, sweeping gas pervaporation, and vacuum pervaporation were applied in ABE fermentations and are reviewed elsewhere (Amiri, 2020; Friedl, 2016; Núñez-Gómez et al., 2014; Vees et al., 2020).

Sustainable sources of syngas include process gases from steel mills and the products of organic waste gasification plants. The conversion of such resources to value-added chemicals could help to sustainably offset industrial greenhouse gas emissions.

Syngas, a mixture of CO, CO₂ and H₂ in differing amounts, can be metabolized by a heterogeneous group of bacteria known as acetogens. Unlike chemical catalysts, these bacteria can metabolize syngas under

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ambient conditions and can accommodate syngas substrates varying in composition and impurity levels (Daniell et al., 2012; Griffin and Schultz, 2012). Acetogens metabolize syngas via the Wood-Ljungdahl pathway, also known as the reductive acetyl-CoA pathway. Two molecules of C1 substrate are used to form 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 (Müller, 2003).

Syngas fermentation with solventogenic acetogens usually involves two production phases. The first is an exponential growth phase that involves acetogenesis and is directly linked to energy conservation (Bertsch and Müller, 2015; Müller, 2003; Schuchmann and Müller, 2014). Once acetate and other primary products accumulate, the bacteria shift from acetogenesis to solventogenesis. During solventogenesis, the primary acidic products are reduced to the corresponding alcohols. Some acetogens, including *C. carboxidivorans* P7, can extend the carbon chain to synthesize the C4 products butyrate and butanol, and the C6 products caproate and hexanol (Figure 1) (Liou et al., 2005; Phillips et al., 2015; Shen et al., 2020; Veas et al., 2020).

The ability of *C. carboxidivorans* P7 to produce caproate and hexanol from syngas can be enhanced by medium and process optimization (Doll et al., 2018; Phillips et al., 2015; Shen et al., 2017). For example, the optimization of trace element composition significantly improved bacterial growth, leading to the currently highest reported titer of 1.33 g/L (13.0 mM) hexanol in combination with a temperature shift to 25 °C after an initial growth phase at 37 °C (Shen et al., 2017). A subsequent study revealed that Wood-Ljungdahl pathway associated genes were expressed more strongly at 37 °C while incubation at 25 °C promoted higher expression of genes involved in butanol and hexanol production (Shen et al., 2020).

Furthermore, a two-step fermentation process has been developed to overcome the limitations of biphasic growth (Doll et al., 2018). Generally, most cell growth is associated with acetogenesis and growth is inhibited as the medium becomes more acidic, triggering the switch to solventogenesis. In the two-step fermentation process, the first fermenter is held at pH 6 for acetogenesis, allowing the accumulation of biomass, acetate and ethanol. The medium in the first fermenter is renewed and some of the broth is flushed to a second fermenter with cell retention at



Figure 1. Schematic overview of butanol and hexanol production from acetyl-CoA. Abbreviations are: WLP = Wood-Ljungdahl pathway, CoA = Coenzyme A, Fd = Ferredoxin, NAD = Nicotinamide adenine dinucleotide, P_i = inorganic phosphate, ATP = Adenosine triphosphate.

pH 5 for solventogenesis. This allows longer fermentation runs because the first fermenter continuously provides cells for the formation of products with longer carbon chains (Doll et al., 2018).

One factor limiting syngas fermentation is the toxicity of the products. As reported for *C. carboxidivorans* P7 grown at 30 °C with CO as a sole carbon and energy source, a 50 % decrease in final biomass was caused by either 14–14.50 g/L butanol or 35 g/L ethanol. IC₅₀ values were deduced for initial growth rates in the presence of ethanol (5–10 g/L) and butanol (20–25 g/L), and the drop in growth rate was even more pronounced when a mixture of alcohols was used, indicating greater adverse effects due to co-toxicity (Fernandez-Naveira et al., 2016). In another study, the IC₅₀ value of butanol for *C. carboxidivorans* P7 grown at 25 °C with a gas mixture of 32 % CO, 32 % H₂, 28 % N₂ and 8 % CO₂ was 4.12 g/L, with complete inhibition at 13.92 g/L (Ramio-Pujol et al., 2018). The same authors also calculated the IC₅₀ value for butanol (6.36 g/L) using datasets from the previous study (Fernandez-Naveira et al., 2016; Ramio-Pujol et al., 2018).

Although the effect of acids during fermentation is well understood, and toxicity data are available for both ethanol and butanol, there are no data concerning the potential toxicity of hexanol toward *C. carboxidivorans* P7 (Fernandez-Naveira et al., 2017). The IC₅₀ of hexanol (LogP_{octanol/water} 2.03) is expected to be even lower than the values reported for butanol (LogP_{octanol/water} 0.88) and ethanol (LogP_{octanol/water} -0.31) (Ritter et al., 1995), given that the toxicity of short-to-medium chain length alcohols increases as the chain gets longer due to the corresponding nonpolar characteristics of the carbon chain, as reported for *Escherichia coli* (Wilbanks and Trinh, 2017). Growth of the model acetogen *C. ljungdahliae* has already been tested in the presence of hexanol, with 10 mM hexanol (1 g/L) causing slight growth impairment and ≥50 mM (5 g/L) causing complete growth inhibition (Lauer et al., 2018).

Product inhibition and toxicity during alcohol production can be avoided by the use of (Z)-octadec-9-en-1-ol (oleyl alcohol) as a biocompatible extraction solvent, as reported for ABE fermentation (Roffler et al., 1988; Veas et al., 2020), other hydrophobic products (Jeon et al., 2013), and syngas fermentation (Haas et al., 2018). This organic alcohol is not soluble in water (LogP_{octanol/water} 7.50) and thus not toxic to cells, but efficiently extracts toxic organic products such as short-to-medium chain length alcohols. Hydrophobic extraction solvents 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 in water 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. In this study the toxic effects of hexanol towards *C. carboxidivorans* P7 were investigated and hexanol production was increased by the use of oleyl alcohol as an extraction solvent.

2. Materials and methods

2.1. Chemicals, strains and bacteria cultivation

All chemicals were supplied by Sigma-Aldrich Chemie (Merck, Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany). All gases were supplied by Westfalen (Münster, Germany). Type strains of *C. carboxidivorans* P7 (DSM 15243) and *C. ljungdahliae* (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 dihydrate and 1 g/L yeast extract. Trace elements and vitamins were prepared as described by ATCC, and 2 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 (5 % H₂, 10 % CO₂ and 85 % N₂). L-cysteine (final concentration: 0.75 g/L) was added as a reducing agent. An optimized trace element composition for alcohol production was used to determine IC₅₀ values, for hexanol production

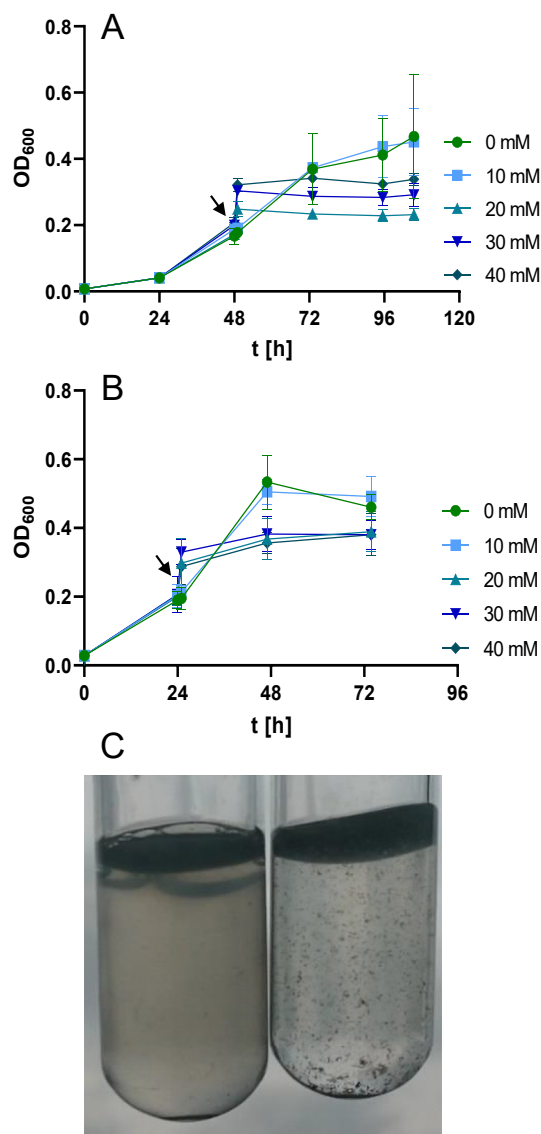


Figure 2. Acute hexanol toxicity in (A) *C. ljungdahliae* and (B) *C. carboxidivorans* P7 at 37 °C. Cells were grown in anaerobic roll tubes with rubber stoppers on syngas (CO, CO₂ and H₂ in equal parts) in modified minimal medium. After definitive growth was observed at OD₆₀₀ = 0.2, hexanol was added (indicated by the arrow) at the indicated concentrations and the gas phase was renewed. Each curve shows a representative experiment from three independent experiments each with three cultures per hexanol concentration (n = 3 ± SD). (C) Macroscopic agglomeration effects of 40 mM hexanol on *C. carboxidivorans* P7 after 3 days after hexanol addition (right) compared to the control without hexanol (left).

and extraction experiments, and for membrane fatty acid analysis (Shen et al., 2017).

2.2. 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 syngas (33.3 % CO₂, 33.3 % CO and 33.3 % H₂) as the growth substrate. Adapted cells were used to inoculate 5-mL medium in 25-mL anaerobic glass tubes (Glasgerätebau Ochs, Bovenden/Lenglern, Germany) with rubber stoppers, and the headspace was filled with syngas of the same composition. The cultures were maintained horizontally at 37 °C with constant agitation at 150 rpm. Cell growth was measured using an HI93703 microprocessor turbidity meter (Hanna Instruments Deutschland, Vöhringen, Germany). This setup allowed faster measuring times and more parallel processing than experiments with serum bottles and samples drawn to measure OD₆₀₀ values. A standard curve was prepared to calculate OD₆₀₀ values from the measured turbidity units for ease of comparison to serum bottle experiments. Once growth was observed (turbidity corresponding to OD₆₀₀ = 0.2), hexanol was added from a pure, sterile, anaerobic stock solution via a 10- μ L SGE syringe (Trajan Scientific and Medical, Ringwood, Australia). After 24 h in the presence of hexanol, 500 μ L samples were drawn and inoculated in fresh medium with fructose as the growth substrate, to ensure the stable regrowth of surviving cells. After recovery for 4 days at 37 °C, the cultures were checked for cell growth. Hexanol extraction with technical grade 85 % oleyl alcohol (Merck) was tested under the same experimental conditions as described above. The medium was supplemented with 100 mM hexanol and 5 % (v/v) oleyl alcohol from sterile, anaerobic stock solutions.

2.3. Determination of minimal inhibitory concentrations (MIC) and IC₅₀ values

The onset of growth inhibition was investigated using the cultivation setup described above but with an optimized trace element composition (Shen et al., 2017) and an adjusted syngas composition (65 % CO, 15 % N₂, 15 % CO₂ and 5 % H₂). The cells were incubated horizontally at the selected temperature, shaking at 150 rpm. 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). Growth rates were normalized against the control without hexanol in the same experiment and were plotted as percentages against the hexanol concentrations added to each culture. Four-factor sigmoidal dose-response curves and IC₅₀ values were calculated using the same program. Fermentations (independent experiments) were carried out five times at 30 °C or four times at 37 °C each comprising three cultures per condition.

2.4. Fed-batch bottle fermentation

Adapted cell cultures were inoculated (OD₆₀₀ = 0.01–0.02) into 25 mL of modified minimal medium with optimized trace elements (Shen et al., 2017) in 250-mL serum bottles (Glasgerätebau Ochs). For the extraction experiments 4 % (v/v) oleyl alcohol were added as above. Cultures were fed with syngas (65 % CO, 15 % N₂, 15 % CO₂ and 5 % H₂) at 1 bar overpressure. The cells were incubated at the selected temperature, shaking at 150 rpm. The growth of cells exposed to the extraction solvent was determined by measuring the OD₆₀₀ after washing the cells by centrifugation (13,000 \times g, 1 min, room temperature) and resuspending them in water. The gas phase was renewed after 1, 2, 3 and 4 days. Samples were drawn using a sterile syringe and stored at –20 °C. At the end of the experiment (8 days), the oleyl alcohol phase and fermentation broth were separated by centrifugation and stored in separate tubes at –20 °C for analysis by gas chromatography.

2.5. Determination of product concentrations by GC/MS

The concentration of fermentation products in the aqueous phase was measured by gas chromatography. 100 μ L of the aqueous phase were mixed with 900 μ L of methanol containing 5.5 mM 1,3-propanediol as external standard. After a second centrifugation step the supernatant was used for measurement (Philipps et al., 2019).

Extraction phase samples were diluted 1:100 in pure methanol and then handled in the same manner as the aqueous samples. 1 μ L of the prepared sample was injected into a GCMS-QP2010S (Shimadzu), evaporated at 200 °C and separated on an InertCap FFAP capillary column (0.25 mm \times 30 m, 0.25 μ m; GL Sciences, Torrance, CA, USA). After an initial hold at 50 °C for 3 min, a temperature gradient (35 °C/min) to 220 °C was applied with a final hold at 220 °C for 2 min for aqueous phase samples. For extraction phase samples the final hold time was increased to 12 min. Ionization was achieved at 1 kV and the total ion count was measured.

2.6. Determination of total fatty acid composition

Cells were inoculated from adapted exponential-phase pre-cultures and grown overnight in 200 mL modified minimal medium with optimized trace elements in 2-L glass bottles with rubber stoppers. The cells were fed with syngas (65 % CO, 15 % N₂, 15 % CO₂ and 5 % H₂) at 0.6 bar overpressure. When the OD₆₀₀ reached 0.4–0.5, the bottles were opened, and the cells were aerobically cooled on ice and harvested by centrifugation (4000 \times g, 15 min, 4 °C) before freeze drying overnight in a VaCO5 device (Zirbus Technology, Bad Grund/Harz, Germany). The membrane fatty acid composition was determined by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) via GC/MS analysis according to the following protocol.

Following saponification, methylation and extraction (Kuykendall et al., 1988; Miller, 1982), the fatty acid methyl esters (FAMES) were analyzed by gas chromatography and detected by flame ionization, with peak identification following the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE, 19711 USA), percentages were calculated by the MIS Standard Software (Microbial ID). Peak identification was confirmed by gas chromatography mass spectrometry (GC-MS) using an Agilent Technologies (Santa Clara, CA, USA) GC-MS 7000D device equipped with an Agilent HP-5ms UI column (30 m \times 250 μ m \times 0.25 μ m). An injection volume of 1 μ L was used with a split ratio of 7.5:1 and a helium gas flow of 1.2 ml/min was applied. The oven temperature was set to an initial temperature of 170 °C, increasing at 3 °C/min to 200 °C, then at 5 °C/min to 270 °C, then at 120 °C/min to 300 °C, followed by a hold for 2 min. The inlet temperature was initially adjusted to 170 °C and then increased linearly at 200 °C/min to 350 °C followed by a hold for 5 min. The MS parameters were set as follows: auxiliary temperature was 230 °C, source temperature 230 °C, electron impact ionization 70 eV with mass range of m/z 40–600 or 40–800. Peaks were identified based on retention time and mass spectra. To confirm the position of double bonds, samples were derivatized to corresponding dimethyl disulfide adducts (Moss and Lambert-Fair, 1989).

2.7. Comparison of different extraction solvents

Cells were cultivated as described above for the fed-batch experiments. A reduced media volume of 4.5 mL was supplemented with 10 % or 20 % oleyl alcohol, sunflower oil or corn oil in 250-mL bottles to avoid the need for gas renewal during experiments without growth curves. Bottles were inoculated from exponential pre-cultures to OD₆₀₀ = 0.1–0.2. Sunflower seed oil from *Helianthus annuus* (88921-250ML-F) and corn oil (C8267-500ML) were both purchased from Sigma-Aldrich Chemie, filter-sterilized and stored under anoxic conditions for several days before use.

3. Results and discussion

3.1. The acute toxicity of hexanol

To determine whether the ability to produce hexanol confers increased resistance to this product, growth of the natural hexanol producer *C. carboxidivorans* P7 was compared to that of the model acetogen *C. ljungdahlii*, which does not produce hexanol. *C. ljungdahlii* is better characterized, and genetic manipulation is considered routine (Leang et al., 2013; Philipps et al., 2019), so a significantly higher tolerance toward hexanol would allow this organism to be used for hexanol production rather than the comparatively less well known natural hexanol producer *C. carboxidivorans*. Acutely lethal hexanol concentrations were determined in 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 (0, 1, 2, 3 or 4 g/L) hexanol during the exponential growth phase.

Interestingly, despite the difference in metabolic capability, both strains displayed similar tolerance for hexanol under the conditions tested (Figure 2A,B). There was no immediate effect on the growth of either strain in the presence of 10 mM hexanol, but exposure to ≥ 20 mM caused an increase in turbidity immediately after the hexanol was added and the turbidity remained stable thereafter. This observed increase in turbidity after hexanol addition only occurred in the presence of cells and not in sterile medium. This indicates a direct interaction between the alcohol and the cells rather than an optical effect caused by the addition of hexanol to the medium. Furthermore, gas consumption ceased in cultures with hexanol concentrations ≥ 20 mM, confirming the significant inhibition of growth and syngas utilization.

To determine whether the cells were killed or merely dormant, 500- μ L aliquots were removed 24 h after hexanol addition and transferred to hexanol-free medium with fructose as the growth substrate to determine 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 regrow within a 4 day period. 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 agglomeration of the cells was observed at the end of the cultivation period (Figure 2C), indicating that hexanol might have a deleterious effect on the cell membrane. Cell agglomeration was also observed during fed-batch bottle fermentations after 3–4 days and has been reported in other studies focusing on alcohol production with *C. carboxidivorans* (Shen et al., 2017) and could be mediated by incubation at lower temperatures (Shen et al., 2020).

Intuitively, one might expect greater hexanol tolerance in a natural producer than in an organism without natural exposure to this compound. However, the similar sensitivity observed for both species probably reflects the natural environment of *C. carboxidivorans* P7, which was first isolated from an agricultural settling lagoon (Liou et al., 2005). In this environment, hexanol titers would not reach inhibitory levels due to the diffusion of the product. Hexanol production by *C. carboxidivorans* P7 in its natural environment is therefore unlikely to impose selection pressure for improved tolerance. The similar hexanol tolerance in both species led to the decision to focus on the natural hexanol producer *C. carboxidivorans* for further characterization.

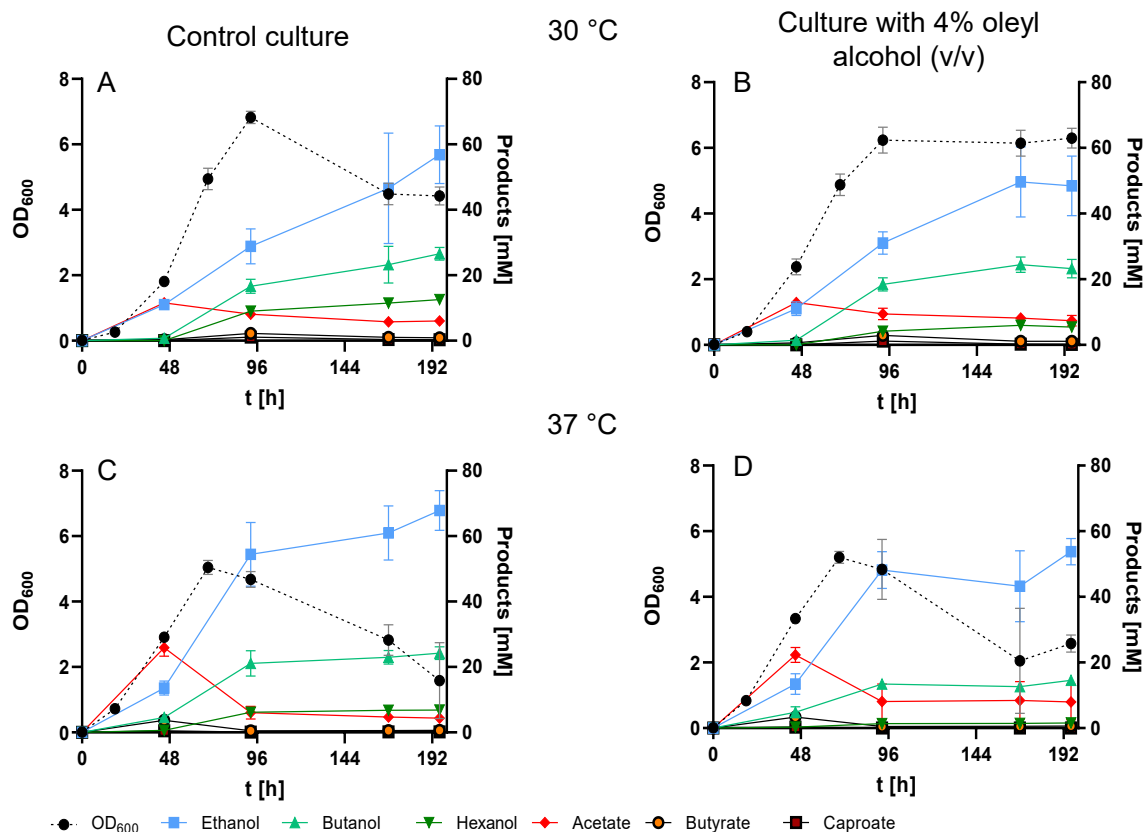


Figure 3. *C. carboxidivorans* P7 growth curves and products in the aqueous phase with in situ extraction. Cells grown at 30 °C in modified minimal medium without extraction (A) and in the presence of 4 % (v/v) oleyl alcohol as an extraction solvent (B). Cells grown at 37 °C in modified minimal medium without extraction (C) and in the presence of 4 % (v/v) oleyl alcohol as extraction solvent (D). The syngas composition was 65 % CO, 15 % N₂, 15 % CO₂ and 5 % H₂. Each curve shows a representative experiment from at least three independent experiments, each with three cultures per condition tested (n = 3 ± SD).

3.2. Hexanol titers at the onset of inhibition and calculation of IC₅₀ values

Having evaluated the acute toxicity of hexanol, the minimal inhibitory concentration (MIC) and IC₅₀ for *C. carboxidivorans* P7 were determined next. The MIC is the lowest hexanol concentration that prevents growth, whereas the IC₅₀ is the hexanol concentration at which the initial growth rate is reduced by 50%. Given the reported positive effect of lower incubation temperatures on alcohol production (Ramio-Pujol et al., 2015; Shen et al., 2017, 2020), both parameters (MIC and IC₅₀) were determined at the standard growth temperature of 37 °C and also at 30 °C. The lower temperature was chosen to achieve an acceptable tradeoff between potentially increased hexanol tolerance at lower temperatures and faster growth at higher temperatures.

Cells were grown in anaerobic glass tubes containing modified minimal medium and syngas as a growth substrate, as described for the acute toxicity experiments in section 3.1. To determine precise inhibitory titers, hexanol was added before inoculation to avoid the increase in turbidity observed in the experiment shown in Figure 2, which might otherwise lead to artefactual higher IC₅₀ values. For experiments at 30 °C, the medium was supplemented with 0, 12, 14, 15, 16, 18, 20 or 22 mM hexanol. Exponential growth began immediately after inoculation. On the second day at 30 °C in the presence of hexanol, the growth rates decreased rapidly and a near linear growth profile was observed (Supplementary Figure 1). This suggests that the toxicity of hexanol increases with longer exposure times or that the synthesis of natural products (mostly acetate and ethanol at this stage) increases stress due to co-toxicity.

Both the initial growth rates and final biomass yields were lower in cultures with higher hexanol concentrations. Cultures supplemented with 22 mM hexanol did not show significant growth, and cultures supplemented with 20 mM hexanol doubled once on the first day and then stagnated (MIC = 20–22 mM hexanol). After two days of growth, cultures containing 12 mM hexanol achieved only ~50% of the biomass yield of the control without added hexanol, confirming that significant growth inhibition occurred even at low hexanol titers. The IC₅₀ for hexanol at 30 °C was 17.5 ± 1.6 mM based on normalized initial growth rates.

At 37 °C, the impact of 0, 7, 10, 12, 13, 14 and 15 mM hexanol was investigated and an IC₅₀ value of 11.8 mM ± 0.6 mM was calculated. At titers ≥15 mM, no growth was observed at 37 °C (MIC = 15 mM hexanol). Accordingly, a shift from 37 °C to 30 °C leads to a 48% increase in hexanol tolerance while doubling times increased only by 33% from 7.5 ± 0.2 h at 37 °C to 10.1 ± 1.0 h at 30 °C. Because growth at the lower temperature also increased biomass yields, fermentation at 30 °C appears to offer a favorable tradeoff between growth rate and product tolerance.

The IC₅₀ of hexanol for *C. carboxidivorans* determined in this study was much lower than the values previously reported for ethanol (20–25 g/L) and butanol (4.12–6.36 g/L) (Fernandez-Naveira et al., 2016; Ramio-Pujol et al., 2018). The higher toxicity of longer-chain alcohols is anticipated because such products are less polar, and thus interact more strongly with lipid membranes (Weber, 1995; Wilbanks and Trinh, 2017). Hexanol toxicity in *E. coli* mainly reflects the effect of this alcohol on membrane fluidity (Ingram and Vreeland, 1980). The macroscopic agglomeration of *C. carboxidivorans* P7 cells in the presence of higher

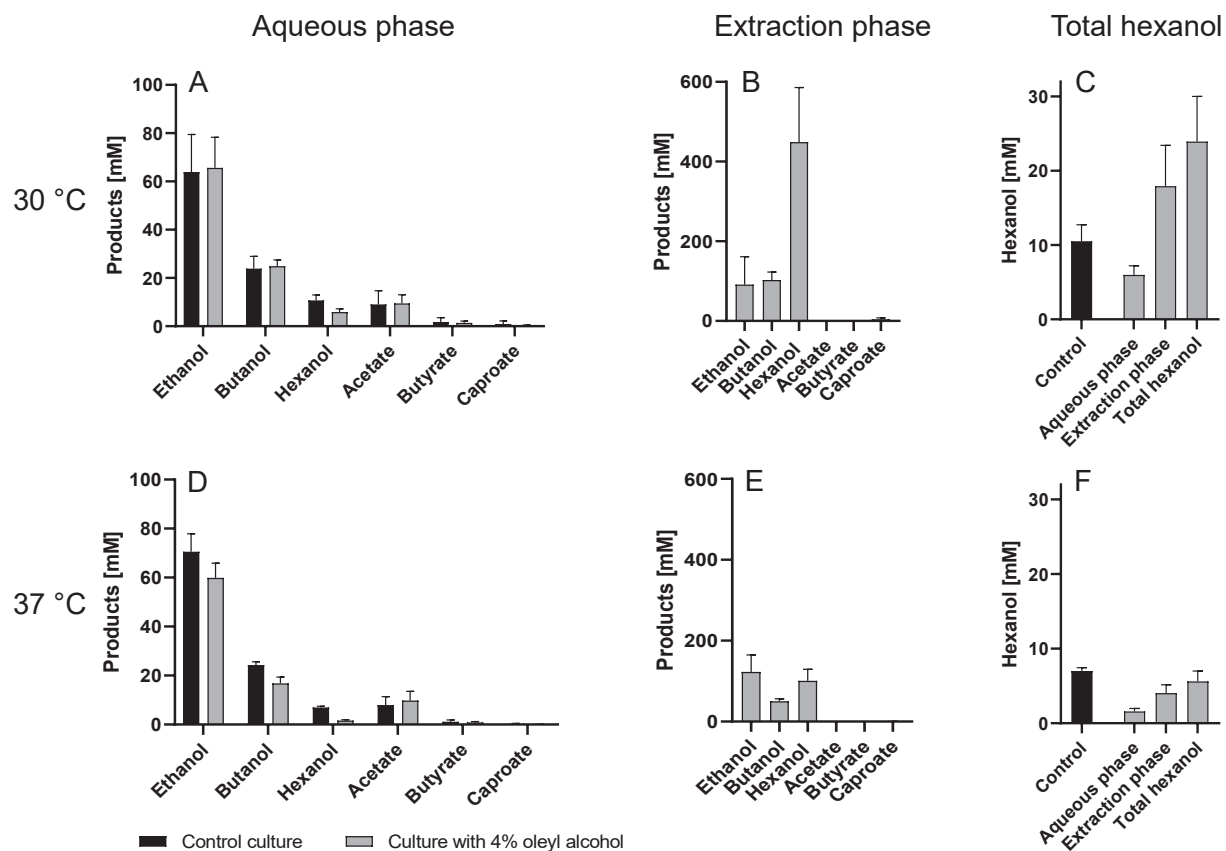


Figure 4. Final products of *C. carboxidivorans* P7 in the presence of 4% oleyl alcohol as an extraction solvent. Cells were grown for 8 days in modified minimal medium with syngas as a growth substrate (65% CO₂, 15% N₂, 15% CO₂ and 5% H₂). Black bars indicate the control culture (no oleyl alcohol) and gray bars indicated cultures in the presence of 4% (v/v) oleyl alcohol. Product titers of cultures grown at 30 °C as measured in the aqueous phase (A) and in the extraction phase (B). Product titers of cultures grown at 37 °C as measured in the aqueous phase (D) and in the extraction phase (E). Total hexanol production of cultures grown at 30 °C (C) and grown at 37 °C (F). Hexanol titers measured in oleyl alcohol were normalized to the total culture volume to allow for comparison with titers measured in the aqueous phase. Hexanol titers of cultures grown without oleyl alcohol are shown as the negative control (black bars). Data are means ± standard deviations of at least three independent experiments, each comprising three cultures per condition tested.

concentrations of hexanol indicates a similar mode of toxicity in *Clostridium* spp.

Both *C. carboxidivorans* P7 and *C. ljungdahlii* appear less sensitive to hexanol than *E. coli*, the latter showing 45 % inhibition of exponential growth in the presence of 0.625 g/L (6.3 mM) hexanol (Willbanks and Trinh, 2017) whereas *C. carboxidivorans* P7 began to experience growth inhibition at approximately double this concentration (10–12 mM) in our experiments. The sensitivity of *C. carboxidivorans* toward hexanol is similar to values reported for other *Clostridium* spp. as well as methanogens. The IC_{50} of hexanol for an enrichment culture of methanogens was 1.5 g/L (Blum and Speece, 1991), and in *C. acetobutylicum* the MIC of hexanol was 1.4 g/L (Lepage et al., 1987).

3.3. Oleyl alcohol avoids product toxicity

Oleyl alcohol has been widely used as an extraction solvent in ABE fermentation and recently also in syngas fermentation (Haas et al., 2018), but has not yet been used in syngas fermentation with *C. carboxidivorans*. Cells were cultivated in modified minimal medium with syngas as above, but with added 5 % (v/v) oleyl alcohol and 100 mM hexanol. *C. carboxidivorans* P7 was able to grow robustly, confirming that oleyl alcohol does not impair growth with gaseous substrates and is able to detoxify the medium with hexanol titers of at least approximately twice the concentration soluble in water. However, in the presence of cells 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 OD_{600} values directly. For subsequent experiments, cells were therefore harvested and washed by centrifugation and resuspension before OD_{600} values were determined.

3.4. In situ 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. *C. carboxidivorans* P7 cells were inoculated into 250-mL serum flasks containing 25 mL medium at either 30 °C or 37 °C and were fed with syngas (65 % CO , 15 % CO_2 , 15 % N_2 and 5 % H_2) at 1 bar overpressure, with gas-phase renewal every 24 h for 5 days. Maximum biomass at 30 °C was reached after 5 days of growth with OD_{600} values of 6.3 ± 0.6 in the control (Figure 3A) and 6.2 ± 0.7 in the culture containing 4 % (v/v) oleyl alcohol (Figure 3B), confirming our earlier observation that oleyl alcohol does not affect growth behavior. At 37 °C, maximum biomass was reached one day earlier with OD_{600} values of 4.9 ± 0.3 in the control (Figure 3C) and 5.4 ± 0.6 in the cultures containing oleyl alcohol (Figure 3D). Although growth was faster at the higher temperature, incubation at 30 °C led to the formation of more biomass. Furthermore, maximum OD_{600} values at 30 °C remained stable for several days in the presence of oleyl alcohol but decreased in the absence of the solvent, confirming its detoxifying effect. At 37 °C however, OD_{600} values decreased both in the presence and absence of oleyl alcohol.

Under all conditions tested, cells produced mostly acetate and ethanol, and later butyrate and butanol during the exponential growth phase. As expected, products were initially generated faster and in higher amounts at 37 °C compared to 30 °C. Hexanol production started during the late exponential to early stationary phase, and the acetate and butyrate titers decreased in all cultures. The final hexanol titers in the aqueous phase after 8 days at 30 °C were 10.5 ± 2.2 mM in the control culture and 6.0 ± 1.2 in the culture containing 4 % oleyl alcohol, whereas the titers of all other fermentation products measured in the aqueous phase were not influenced by the extraction solvent (Figure 4A). In the oleyl alcohol phase, the hexanol concentration was 448 ± 130 mM (Figure 4B), corresponding to 17.9 mM normalized for the culture volume. When added to the 6.0 mM hexanol in the aqueous phase, the overall hexanol titer was 23.9 mM, representing a nearly 2.5-fold increase compared to the control without oleyl alcohol (Figure 4C). Three

quarters (75 %) of the total hexanol were found in the oleyl alcohol phase, corresponding to a concentration factor of approximately 75-fold over the aqueous phase. In addition to hexanol, the oleyl alcohol phase contained 102 ± 20 mM butanol, 126 ± 43 mM ethanol and traces of caproate. Neither acetate nor butyrate was present in the extraction phase.

At 37 °C, 7.0 ± 0.5 mM hexanol was produced in the absence of oleyl alcohol, but only 5.6 ± 1.3 mM was produced in its presence, with 1.6 ± 0.4 mM of the total hexanol located in the aqueous phase and 101 ± 27 mM (4.0 ± 1.0 mM normalized to the culture volume) in the extraction phase (Figures 4E,F). The extraction phase also contained 50.4 ± 5.7 mM butanol and 123 ± 40 mM ethanol as well as traces of caproate. Again, neither acetate nor butyrate was present in the extraction phase. The missing positive effect of oleyl alcohol at 37 °C is most likely explained by the initially faster production of acetate and ethanol at higher growth temperatures. These compounds inhibit growth but are not removed by the extraction solvent due to their hydrophilic nature and mostly accumulate in the aqueous phase. Since the titers of these products are similar at the final stage at both temperatures investigated and cannot be mediated by extraction, we expect them to be the limiting factor in these bottle fermentations.

These findings not only confirm that oleyl alcohol is an efficient hexanol extraction solvent during the fermentation of syngas, but also demonstrate a positive effect on hexanol production by removing the toxic product from the fermentation broth. Cultivation at 30 °C rather than 37 °C led to a 50 % increase in hexanol titers in the absence of oleyl alcohol and more than a four-fold increase in hexanol production in its presence compared to the equivalent fermentations at 37 °C. A shift toward lower temperatures has already been reported as a viable strategy to alleviate hexanol toxicity and acid crash in *C. carboxidivorans*. In one study the cultivation temperature was shifted from 37 °C to 25 °C during the cultivation (Shen et al., 2017) and in the other the cells were continuously cultivated at 25 °C (Ramio-Pujol et al., 2015). A later study comparing bottle fermentations with temperature shift with fermentations without shift at different temperatures found increased hexanol production with a shift from 37 °C to 25 °C due to initially higher biomass formation (Shen et al., 2020). Continuous cultivation at 30 °C with hexanol extraction renders a temperature shift unnecessary during cultivation and achieves faster growth than at 25 °C. This could facilitate scale up and the development of a continuous industrial process.

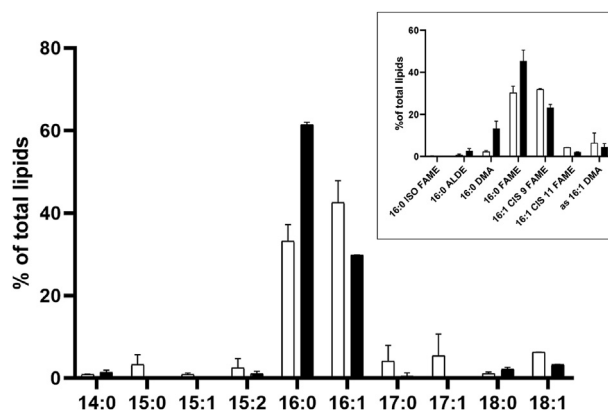


Figure 5. Membrane adaptation of *C. carboxidivorans* grown with syngas at 30 °C (white bars) and 37 °C (black bars). Bars represent sums of percentages of lipid compounds of the same chain length and saturation to show overall trends in composition. Branched chain fatty acids are not shown separately because they represent ≤ 0.8 % of the total per molecular species. Percentages are shown as means \pm standard deviations of two independent experiments with one technical replicate per condition tested. Insert box shows detailed analysis of 16-carbon lipids. Abbreviations: FAME, fatty acid methyl ester, ALDE, aldehyde, DME, dimethylacetal, ISO, iso-branched-chain fatty acid; CIS 9/CIS 11: position of double bond in unsaturated fatty acids. Full details are provided in Supplementary Table 1.

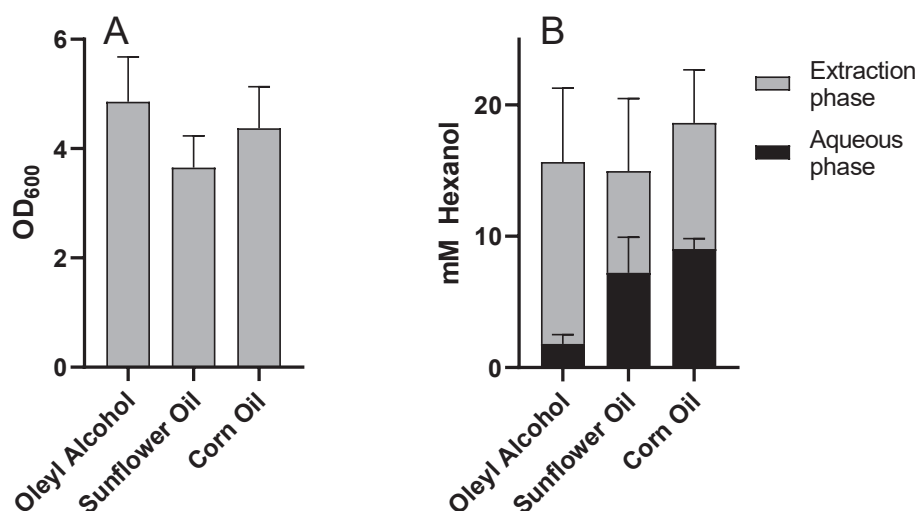


Figure 6. Biomass yields (OD₆₀₀) and hexanol production in the presence of different extraction solvents. Cells were grown for 7 days in modified minimal medium with 1 bar overpressure of syngas as a growth substrate (65 % CO, 15 % N₂, 15 % CO₂ and 5 % H₂). Hexanol titers measured in the extraction phase were normalized to the whole culture volume and are shown as gray bars. Hexanol titers measured in the aqueous phase are shown as black bars. All data are means ± standard deviations of eight cultures from three independent experiments.

3.5. Analysis of cellular membrane fatty acid composition

A fatty acid analysis of the cell membrane of *C. carboxidivorans* was performed to explain the differences in production and growth behavior at the two fermentation temperatures investigated. Several bacteria can adapt their membranes to higher temperatures and/or hexanol by incorporating more saturated fatty acids, counteracting the increase in membrane fluidity and leakage (Ingram and Buttke, 1985). This has been reported in *C. acetobutylicum* (Lepage et al., 1987) and *E. coli* (Berger et al., 1980). In *C. pasteurianum*, the same effect was also observed during *n*-butanol fermentation (Venkataramanan et al., 2014). To reveal a membrane adaptation towards different incubation temperatures, the fatty acid composition of cells grown at 30 °C and 37 °C was determined. Furthermore, to reveal a possible adaptation towards hexanol, the fatty acid composition of cells grown at 30 °C in the presence of 10 mM hexanol was determined.

As expected, when comparing *C. carboxidivorans* grown at 30 °C and 37 °C, a shift toward more saturated fatty acids and overall less diversity in the membrane composition was observed at the higher temperature. At 30 °C, 75.7 ± 6.6 % of the membrane consisted of compounds with a chain length of 16 carbons, but this increased to 91.3 ± 0.4 % at 37 °C. Furthermore, the membrane composition at 30 °C was more diverse with higher proportions of both longer and shorter lipids compared to 37 °C (Figure 5). Among the 16-carbon compounds, a higher proportion of saturated lipids (mostly palmitic acid and 16:0 vinyl ether) was detected at 37 °C, but a higher proportion of mostly 16:1 *cis* 9 and 16:1 *cis* 11 unsaturated lipids was detected at 30 °C. The proportion of saturated lipids shifted from 42.6 ± 1.9 % at 30 °C to 65.7 ± 0.4 % at 37 °C with a correlated decrease mostly in monounsaturated lipids. The only polyunsaturated fatty acid detected was 15:2 (2.5 ± 1.6 % at 30 °C and 1.1 ± 0.5 % at 37 °C). Cyclic or branched chain fatty acids were only found at concentrations of ≤0.8 % per molecular species.

Most interestingly, the *C. carboxidivorans* cell wall lipid composition did not appear to adapt in the presence of 10 mM hexanol (Supplementary Table 1). The observed lack of membrane adaptation may reflect the intrinsic inability of *C. carboxidivorans* to adapt to higher concentrations of hexanol, or adaptation may be possible but the mechanism may not be dependent on lipids and thus was not detected in our experiments (for example adaptation may involve membrane proteins). Finally, even though the cells were adapted to grow in the presence of hexanol before

fatty acid analysis, long-term adaptation to higher titers may lead to different findings in terms of membrane composition.

3.6. Utilization of plant oils as extraction solvents

Oleyl alcohol can be produced by the hydrogenation of oleic acid esters derived from animal or plant oils (Bouveault and Blanc, 1904) and has already been widely used as an extraction solvent in fermentations with *Clostridium* spp. Ideal properties for extraction solvents used during fermentation include biocompatibility, stability, selectivity for the targeted product, and low bulk price. The direct use of plant oils could reduce the cost of and energy requirements for the production of extraction solvents, leading to the decision to investigate biocompatibility and hexanol extraction efficiency of these oils. Therefore corn oil and sunflower oil were investigated as potential replacements for oleyl alcohol. Both plant oils contained approximately similar amounts of oleic acid (18:1) with 29.1 % for corn oil and 30.6 % for sunflower oil. Furthermore, corn oil and sunflower oil respectively contain 11.8 % and 6.5 % palmitic acid (16:0), 1.6 % and 3.6 % stearic acid (18:0), and 55.4 % and 56.8 % linoleic acid (18:2). Laboratory-grade plant oils were used for these experiments because the precise composition is reported by the supplier, but for large-scale applications there are no strong arguments against the use of bulk oils, which are much less expensive.

Cells were grown in 250-mL anaerobic flasks containing 4.5 mL of medium supplemented with one of the three alternative extraction solvents, and were fed with syngas at 1 bar overpressure. The small culture volume avoided the need to renew the gas phase because only the final hexanol titers and OD₆₀₀ values were investigated. The experiment was conducted twice with a 10 % extraction phase and once with a 20 % extraction phase, but the higher solvent volume did not affect the final hexanol titers. The final OD₆₀₀ values (representing biomass accumulation) were 4.9 ± 0.8 with oleyl alcohol, 3.7 ± 0.6 with sunflower oil, and 4.4 ± 0.8 with corn oil (Figure 6A). Final hexanol titers were similar for all three solvents, but extraction was most efficient in the case of oleyl alcohol (Figure 6B). In experiments with a 10 % extraction phase, 85 ± 2 % of the hexanol was extracted by the oleyl alcohol, but only 41 ± 10 % by the sunflower oil and 43 ± 6 % by the corn oil. This tradeoff between production costs and extraction efficiency will need to be evaluated on a case-by-case basis for different applications, and is highly dependent on the reusability of the extraction solvent during industrial processes,

which was not investigated in this study. For laboratory use, both plant oils were viable as inexpensive alternatives to oleyl alcohol.

3.7. Limitations of the present study

All experiments in this study were performed in fed batch bottle fermentations as a proof of principle. Even though the gas phase was renewed regularly in production experiments to prevent substrate limitations, these conditions do not resemble the conditions in controlled fermentation environments of an industrial process. Parameters like fermentation run times at optimal production parameters, extraction- and downstream processing costs (i.e. product separation after in line extraction) were not the subject of this study and need to be addressed for an economic and ecologic evaluation of this process for efficient scale-up.

4. Conclusions and prospects

C. carboxidivorans was able to convert syngas into hexanol, with maximum titers of 2.4 g/L at 30 °C in the presence of oleyl alcohol, which detoxified the culture medium by extracting hexanol from the aqueous phase. This titer is well above otherwise acutely toxic concentrations, confirming that hexanol production is limited by product toxicity. Extraction had no effect at 37 °C and hexanol displayed increased toxicity at higher temperatures. *C. carboxidivorans* cell membranes adapted to higher temperatures but surprisingly not to hexanol. Plant oils were found to be inexpensive albeit less efficient alternatives to oleyl alcohol for hexanol extraction. Removing the limitation of product toxicity in hexanol production from syngas allows for further optimization of the fermentation process to obtain product yields far beyond otherwise toxic titers. Without toxicity limitation towards the biocatalyst, genetic optimization for higher production rates and reduction of unwanted byproducts (i.e. ethanol and acetate) will lead to a more efficient and economically feasible process.

Declarations

Author contribution statement

Patrick Kottenhahn: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gabriele Philipps, Stefan Jennewein: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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