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Cultivation of aerobic hydrogen-oxidizing bacteria on CO_2 derived from anaerobic digestion and acid fermentation

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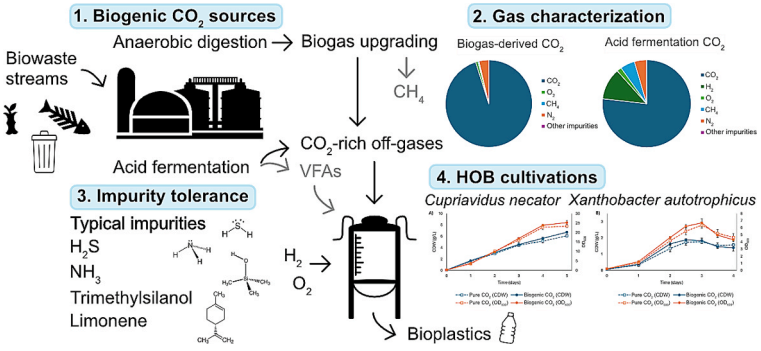
HIGHLIGHTS

- Minimal inhibitory concentrations were determined for key biogas contaminants.
- Biogas-derived CO_2 supported growth comparable to pure CO_2 in bioreactors.
- Polyhydroxybutyrate contents were similar for pure and biogas-derived CO_2.
- Comparable growth was also observed on acid fermentation gases and pure gases.

GRAPHICAL ABSTRACT



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ABSTRACT

Aerobic hydrogen-oxidizing bacteria (HOB) are promising hosts for converting carbon dioxide (CO_2) into biomass and value-added products. However, using biogenic CO_2 derived from anaerobic digestion or acid fermentation remains underexplored, particularly regarding the impact of associated impurities on HOB growth. In this study, CO_2-rich gas streams from these sources were evaluated as carbon feedstocks for *Cupriavidus necator* and *Xanthobacter autotrophicus*. Gas compositions and impurities were analyzed, and minimum inhibitory concentrations (MIC) for typical impurities (H_2S, ammonia, trimethylsilanol, and limonene) were determined. The results show that the MIC for the chosen impurities were relatively high, and HOB growth on biogenic CO_2 was comparable to growth on pure CO_2, indicating that gas purification is not required. Furthermore, bioreactor cultivations demonstrated that biogas-derived CO_2 supports polyhydroxybutyrate (PHB) production at levels

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comparable to pure CO_2. These findings suggest that CO_2 derived from anaerobic digestion and acid fermentation are viable feedstocks for industrial HOB-based production, while providing new insights into impurity tolerance.

1. Introduction

Gas fermentation is an emerging technology where gaseous carbon sources are converted into materials and chemicals by bacteria. Acetogenic bacteria utilize carbon monoxide (CO) or carbon dioxide (CO_2) and hydrogen (H_2) under anaerobic conditions for growth whereas methanotrophs oxidize methane as their source of carbon and energy. A third class of industrially relevant gas fermenting bacteria is aerobic hydrogen-oxidizing bacteria (HOB), which fix CO_2 using H_2 as an energy source and electron donor, and oxygen (O_2) as the terminal electron acceptor.

Production of protein-rich bacterial biomass for food and feed by gas fermentation of HOB is gaining momentum. Companies such as Solar Foods (Finland), Aerbio (Denmark), and Air Protein (USA) are developing industrial-scale protein production using HOB as hosts (Nyyssölä et al., 2022). In addition, HOB have been studied widely for the production of materials, fuels, and chemicals from gases (Brigham, 2019; Donati and Johnson, 2025). An advantage of HOB over CO_2 fixing phototrophs, such as algae and plants, is that they do not need large surface areas for growth. Production takes place in bioreactors, reducing water use and eliminating the need for arable land. High cell concentrations, up to 91 g/L cell dry weight (CDW) with 68 % of polyhydroxybutyrate (PHB), have been achieved in bioreactor cultivations of the most studied HOB, *Cupriavidus necator* (Tanaka et al., 1995). The highest maximum growth rates reported for *C. necator* range from 0.12 1/h (Yu and Munasinghe, 2018) to 0.42 1/h (Ishizaki and Tanaka, 1990).

To scale HOB-based processes, sustainable and affordable sources of H_2 and CO_2 are needed. Any CO_2 emission-free technology for electricity production, such as solar, wind, hydropower, and nuclear, can be harnessed for splitting water into H_2 and O_2 by electrolysis, allowing the utilization of excess energy available during peak production. For CO_2 supply, several sourcing options exist. CO_2 can be separated directly from air by Direct Air Capture (DAC) technologies, with the benefit that the location of the facility does not need to be limited to a particular place, as when point sources are used. However, with the current price (250–600 € per ton), DAC extracted CO_2 is apparently too expensive for use as a feedstock (Decker, 2025). Industrial off-gases have also been suggested as the source of CO_2. These include gases from cement manufacturing, steel production, alcoholic fermentation, and various chemical processes (Psarras et al., 2017).

Another potential source of CO_2 is anaerobic digestion (AD), in which microbes break down organic materials in the absence of oxygen to produce biogas. AD is one of the most widespread bioprocesses globally and is used for treating a range of organic waste and side streams from e.g. agriculture, food industry, and municipalities. In addition to CH_4 (50–75 %), biogas contains a significant fraction of CO_2 (25–50 %) (Fagerström et al., 2018). This CO_2 is typically released into the atmosphere after it is separated during upgrading the biogas to biomethane. Because the carbon in biogas originates from recently fixed biomass rather than fossil sources, using the abundantly available biogas-derived CO_2 as the feedstock can reduce the carbon footprint of gas-fermentation based production.

AD can alternatively be operated as acid fermentation (AF), where AD is halted before methanogenesis. This results in the generation of H_2, CO_2, with possible traces of methane, as well as volatile fatty acids (VFAs), which are versatile platform chemicals that can be used for synthesis of biobased plastics, such as polyhydroxyalkanoates (PHA), solvents and other value-added chemicals (Sabaratnam and Hassan, 2012; Słupek et al., 2019; Wainaina et al., 2019). Compared to biogas, the gas mixtures from AF are less studied in terms of composition. The few reported results show significant variation, with CO_2 in the range of 25–85 % and H_2 in the range of 12–65 % (Amulya and Venkata Mohan, 2022; Costa et al., 2023; Lauri et al., 2021; Moscoviz et al., 2018; Park et al., 2005). Most studies focus on optimizing the production of VFAs and/or H_2 while overlooking the produced biogenic CO_2. The gas mixture containing both CO_2 and H_2 could serve as a valuable substrate for gas fermentation of HOB.

HOB research has almost solely relied on pure laboratory gases as the substrates for growth, whereas industrial gases have rarely been used for cultivation. However, some recent studies have tested more realistic conditions. Co-cultures of HOB and methane-oxidizing bacteria have been cultivated on biogas-based gas supplemented with H_2 and O_2 (Kerckhof et al., 2021), and mixed HOB cultures have been grown on a simulated syngas mixture with varying CO and H_2S levels as inhibitors (Pelagalli et al., 2024). The impurities and their concentrations of industrial CO_2 vary depending on the source and isolation method. In biogas, a range of compounds, such as H_2S and other sulfur compounds, siloxanes, halogenated compounds, and NH_3, have been reported (Czatzkowska et al., 2020). Impurities in acid fermentation gas have been studied less, but NH_3, H_2S, and CO may be present (Lauri et al., 2021; Słupek et al., 2019). In a recent publication, *C. necator*, was cultivated autotrophically on raw biogas supplemented with H_2 and O_2. CO_2 was converted into the bioplastic PHB, thereby reducing the CO_2 fraction and consequently increasing the relative CH_4 content of the gas. The authors reported comparable growth on biogas as on pure gases, with no apparent growth inhibition (Serna-García et al., 2024). Similarly, another study reported that *C. necator* cell mass cultivated on glucose could produce PHB from CO_2 isolated from biogas with the same efficiency as from pure CO_2 (Garcia-Gonzalez and De Wever, 2017). However, given the significant variability in biogas composition, it should be evaluated how different impurities in the gas streams affect microbial growth. In addition, it is important to validate non-toxicity of biogases and isolated CO_2 fractions from a range of sources.

As impurity tolerance remains poorly understood, it is important to consider potential differences between HOB species. Several industrially relevant HOB species have been identified. *C. necator* is widely used for PHA production and is considered the model organism for HOB (Morlino et al., 2023). Members of the genus *Xanthobacter* are emerging HOB and a *Xanthobacter* strain has been patented for single-cell protein production by Solar Foods (Holmstörm and Pitkänen, 2026). *Xanthobacter* species, such as *X. autotrophicus,* have also been reported to produce PHA up to 60 % CDW and possess the additional advantage of nitrogen fixation capability (Morlino et al., 2023), which is absent in *C. necator*. Despite these promising traits, no studies have been conducted with biogas-derived CO_2 with *Xanthobacter* spp. In addition to pure cultures, mixed HOB populations are also being explored as potential single-cell protein producers (Pelagalli et al., 2024). However, the use of pure cultures in the present study provides a simpler framework for evaluating the effects of different impurities. Furthermore, in food applications regulatory approval is typically given to a single, defined production organism, which may be more complicated for mixed cultures.

The aim of this study was to assess the suitability of biogenic CO_2 derived from AD and AF as carbon sources for two industrially relevant HOB, *C. necator* and *X. autotrophicus*, and to examine the impact of typical gas impurities on their growth. Growth was evaluated using defined concentrations of individual synthetic impurities as well as CO_2-rich gas samples obtained from biogas upgrading and AF. This study demonstrates that both CO_2 isolated from biogas as well as the gaseous phase of AF of mixed biowaste can be used for cultivation of HOB and that typical biogas impurities are tolerated at relatively high

concentrations by the HOB studied. The results suggest that gas fermentation of HOB could be integrated with biogas-upgrading facilities that vent biogenic CO_2 and with AF processes that co-produce CO_2 and H_2. Potential applications include single-cell protein for food and feed and bioplastics (PHA/PHB), where raw, unrefined CO_2 feedstock is essential for scalable and economically viable industrial production.

2. Materials and methods

2.1. Microbial strains, culture media, and cultivation conditions

Cultivations of the HOB *Cupriavidus necator* E-173524 (VTT culture collection, also available as NCIMB 11599), *Xanthobacter autotrophicus* 7C (DSM 432), and *Xanthobacter autotrophicus* SF (DSM 2267) were carried out at 30 °C in a modified DSMZ81 mineral medium at pH 6.8 (see composition in supplementary materials).

2.2. Cultivations in the presence of synthetic impurities

Impurities in biogenic gas streams are highly diverse. Representative inhibitory compounds were therefore selected from the following categories commonly found in biogas: sulfide and sulfur compounds (H_2S, added as Na_2S), terpenes (limonene), silicon compounds (trimethylsilanol), and nitrogen compounds (ammonium, added as $(NH_4)_2SO_4$). These groups were selected to cover the main classes of biogas inhibitors relevant to microbial growth. All selected compounds have been reported in various biogas streams (Bragança et al., 2020). Aromatic and halogenated compounds were not included, as their expected concentrations in biogas are generally low (Kajolinna et al., 2025; Werkneh, 2022).

Cultivations in the presence of synthetic impurities were conducted in rubber-stoppered test tubes (25 mL) with a culture volume of 3 mL in autotrophic and heterotrophic conditions. For heterotrophic cultivations, fructose was added at a concentration of 20 g/L for precultures and 10 g/L for the main cultures. The gas mixture for autotrophic cultivations consisted of 10 vol-% CO_2, 80 vol-% H_2, and 10 vol-% O_2 (Wiegel, 2006). The tubes were shaken at 200 rpm in a horizontal position. Optical density at 600 nm (OD_{600}) was measured using a spectrophotometer modified for test tube measurements until an absorbance of 2.0 was achieved. The values from the measurements were corrected for a light path of 1 cm using the equation $0.5485 \cdot OD_{600}(\text{measured}) + 0.0414$ ($R^2 = 0.9984$), determined by measuring absorbances of a dilution series of a cell suspension of *C. necator* with a standard spectrophotometer. Each impurity was tested individually at a minimum of five concentrations in triplicate (see supplementary material). Precultures were grown to an exponential growth phase and inoculated to an OD_{600} of around 0.1. Minimum inhibitory concentrations (MIC) were determined after 48 h of cultivation, defined as OD_{600} not exceeding 0.3 (Tiwari et al., 2023; Wiegand et al., 2008). This threshold was selected to ensure that only cultures exhibiting clear growth, corresponding to at least two cell divisions, were considered non-inhibited. The 48-h time point was chosen as control cultures reached stationary phase in 24 h, allowing sufficient time for growth under inhibitory conditions. Growth beyond 48 h was not assessed.

2.3. Bacteriophage enrichment from biogas digestate

Bacteriophage enrichment was performed to evaluate whether digestate-derived samples contain phages capable of infecting the studied strains. Although bacteriophages are rarely considered in the context of gas-derived feedstocks, their presence cannot be excluded, and enrichment is a standard approach to increase the likelihood of detecting low-abundance phages (Artawinata et al., 2023). Digestate samples were collected from three Finnish biogas plants treating municipal biowaste (Mustankorkea, Jyväskylä; Ämmässuo, Espoo; Nevel, Forssa). The samples were transported on ice and processed within 24 h of sampling. Digestate (25 mL) was mixed with an equal volume of twice concentrated tryptic soy broth (TSB) with 4 mM $CaCl_2$ and vortexed. The mixture was centrifuged for 10 min at 10 000 x g, the supernatant was collected, and then recentrifuged for 1.5 h at 50 000 x g. The pH of the resulting supernatant was adjusted to 7.0 with HCl and sterile filtered through a 0.22 µm membrane. Bacteriophage enrichment cultivations were carried out in 5 mL of the filtered digestate supernatant and 5 mL of TSB supplemented with 2 mM $CaCl_2$. The media were inoculated with *C. necator* or *X. autotrophicus* 7C to an OD_{600} of 0.1 and incubated overnight. *Pseudomonas* sp. (DSM 21482) was used as a positive control, with its corresponding bacteriophage Phi6 (DSM 21518) added to the sterile-filtered digestate supernatant at a concentration of $1x10^2$ plaque forming units (PFU)/ml. All *Pseudomonas* cultivations were performed at 25 °C.

Enrichment of bacteriophages was assessed with the standard soft agar overlay method (Van Twest and Kropinski, 2009). Enrichment cultures of *C. necator* and *X. autotrophicus* 7C were centrifuged for 10 min at 10 000 x g and the supernatant was sterile filtered (0.22 µm) to isolate potential phages. For plaque assays, 4 mL TSB soft agar (0.6 %) with 2 mM $CaCl_2$ was mixed with 150 µL of exponentially growing bacteria and 150 µL of phage enriched supernatant, and poured onto TSB agar (1.5 %) plates. Additionally, 10 µL droplets of phage enrichment were spotted on solidified agar. As a control, phage enrichments were heat-inactivated at 95 °C for 10 min and plated similarly. Plates were incubated until growth was visible on the control plates. Phage enrichment cultures were also streaked on TSB plates to confirm that bacterial growth had taken place in the presence of potential digestate inhibitors.

2.4. Bioreactor cultivations with biogas-derived CO_2

A CO_2-rich off-gas from biogas upgrading to CH_4 was used as a carbon source for bioreactor cultivations (CO_2 content of 95.3 vol-%). A 50 L gas cylinder was filled at the Mustankorkea biogas plant (Jyväskylä, Finland), which uses municipal biowaste as a feedstock and employes pressure swing absorption (PSA) as the biogas upgrading method to separate CH_4 from CO_2 (Riboldi and Bolland, 2017). The sampled gas was compressed to 30 bar into the gas cylinder using an oil-free compressor. The composition and impurities of the gas was analysed as described in the supplementary materials.

Batch cultivations were carried out using the 15-vessel Medicel® Explorer Cultivation Unit (Medicel, Espoo, Finland) as previously described with few modifications (Jämsä et al., 2023). Inoculum cultivations were carried out in 20 mL of modified DSMZ81 medium in 100 mL shake flasks. The medium was supplemented with gentamicin (10 µg/ml) for *C. necator* to minimize contamination risk (Boy et al., 2021; Di Bisceglie et al., 2025), and with antifoam (1 ml/L, Struktol J673A) for *X. autotrophicus* SF (Jämsä et al., 2023). Flasks were incubated for three days in a 34.5 L sealed container under continuous gas flow (20 ml/min H_2, 6 ml/min CO_2, 3 ml/min O_2) in a shaker at 137 rpm. Bioreactors, with working volumes of 200 mL, were inoculated to an initial OD_{600} of 0.25 and supplied with a continuous flow of H_2-CO_2-O_2 gas mixture at flow rates of 51.6 ml/min H_2, 6 ml/min CO_2, and 2.4 ml/min O_2. This corresponds to an oxygen concentration of 4 vol-%, which is below the lower explosion limit. The CO_2 concentration was set to 10 vol-% due to reported inhibitory effects of elevated CO_2 on *C. necator* (Amer and Kim, 2023; Repaske et al., 1971). Three parallel bioreactors were run for each gas stream, using either biogas-derived CO_2 or pure CO_2 at stirring rates of 700 rpm and 400 rpm for *C. necator* and *X. autotrophicus* SF, respectively.

Growth was monitored every 12 or 24 h by OD_{600} and cell dry weight (CDW) measurements. For CDW determination, two aliquots of 1.9 mL per reactor were collected and centrifuged. The cells were washed twice with double-distilled water and dried at 105 °C overnight in pre-weighted 2 mL Eppendorf tubes. Due to the low biomass levels of *X. autotrophicus* at the early phase of the cultivation, direct CDW determination was not possible. Therefore, CDW values for the initial

biomass concentrations and at 24 h were estimated from OD_{600} measurements using a conversion factor of 0.31 OD_{600}/CDW. The bioreactor system was not equipped with off-gas analysis and therefore gas consumption rates could not be quantified in this study. At the end of the cultivations, cell samples were collected and freeze-dried for polyhydroxybutyrate (PHB) and elemental nitrogen analysis. Elemental nitrogen was measured following a previously reported method (Nordlund et al., 2018) and multiplied by the common conversion factor of 6.25 to estimate protein content of the cells. Since this is an indirect measure of protein, there may be some associated uncertainty in the estimation. The residual ammonia concentration in the supernatant was analyzed using Ammonia (Rapid) Assay Kit (Megazyme, Ireland).

2.5. Acid fermentation

The substrate for AF was separately collected municipal biowaste (BW) from Forssa, Finland. The BW was manually sorted by removing largest pieces of plastics, metals, glass, and other inorganic material to prevent damage to the knife mill and process equipment. After sorting, the substrate material was crushed with a knife mill (Retsch GM 300, 2000 rpm, 15 s) to achieve homogenous composition. The substrate was divided into portions and stored at −18 °C.

The inoculum was collected from a mesophilic (37 °C) biogas reactor (Nevel, Forssa, Finland) treating separately collected municipal BW. At first, the inoculum was sieved with a 2 x 2 mm filter to remove the non-biodegradable material, such as pieces of plastics and metals. The inoculum was then stored at 37 °C for 1 day, after which it was boiled for 30 mins to inactivate the methanogens (Blasco et al., 2020). After boiling, the inoculum was incubated at 37 °C for approximately 9 days to degrade the remaining organic compounds, according to a pre-incubation method described previously (Angelidaki et al., 2009).

The batch experiment was performed at 37 °C using an automated testing equipment (Bioprocess Control Ltd, Sweden) for 160 h. The samples were prepared in triplicates, each 500 mL sample bottle containing 400 g of liquid mass. The selected ratio of BW substrate and inoculum based on the content of volatile solids (S/I) was 1:1 for AF experiment “BW1” and 2:1 for AF experiment “BW2”. Distilled water was added to reach equal mass in each sample (Tampio et al., 2019). Anaerobic conditions were achieved by flushing the sample bottles with CO_2. CO_2 was applied instead of commonly applied N_2 in order to avoid dilution of the product gas. The proportion of the flushing CO_2 in the final gas product can be estimated to be small, approximately 2–3 %. During the experiment, the volume of total produced gas was determined by the water displacement method. The gas volume was converted into normal conditions (0 °C, 101.32 kPa), according to the ideal gas law. The gases produced from AF were collected in gas bags (Restek, USA). From BW1, gas from one of the triplicates was used for the test tube cultivation experiments. From BW2, gas from all triplicates was combined due to the low volumes produced. The composition and impurities in produced gases were analysed as described in the supplementary materials.

Total solid (TS) and volatile solid (VS) analyses were performed for samples of substrate and inoculum according to the standard SFS 3008 (Suomen standardisoimisliitto, 1990). For the inoculum and BW, TS and VS were 3.33 % and 2.35 %, and 27.62 % and 25.32 %, respectively. A benchtop pH meter (Thermo Scientific, Orion 4-star) was used for pH measurement.

2.6. Cultivations with gas from acid fermentation

Because the volumes of gas produced in BW1 and BW2 were relatively small, cultivations were performed in test tube scale using only *C. necator*. The cultivations were carried out as described in section 2.2. for testing the synthetic impurities, except that samples were withdrawn with a needle and a syringe when the absorbance at 600 nm, measured using the spectrophotometer adapted for test tubes, exceeded 2.0. The absorbances of the samples were then measured using a standard spectrophotometer. For investigating growth on AF gases, the gas mixtures were adjusted to contain gas from AF at a CO_2 concentration of 10 vol-%. Synthetic O_2 and H_2 were added to achieve final contents of approximately 10 vol-% and 80 vol-%, respectively. As a reference, synthetic gas mixtures of similar composition, in which the CH_4 was replaced by N_2, were prepared. The compositions of the gas mixtures are described in detail in Table 3. The cultivations were continued until stationary phase and placed on ice, after which the gas phases were changed to fresh ones and the cultivations continued.

2.7. Determination of PHB by gas chromatography-mass spectrometry

PHB was quantified by gas chromatography-mass spectrometry (GC–MS). About five milligrams of lyophilized biomass was subjected to methanolysis at 100 °C for 140 min in 1 ml chloroform, 50 µl internal standard (3-hydroxybutyric acid), 150 µl sulfuric acid, and 800 µl methanol. After cooling to room temperature, water-soluble components were removed by adding 1 ml of distilled water. The chloroform phase was analyzed by gas chromatography (7890, Agilent) with a HP-FFAP column (19091F-102, Agilent). 3-hydroxybutyric acid was used as a standard and treated similarly to samples.

3. Results and discussion

3.1. Growth of *C. necator* and *X. autotrophicus* in the presence of synthetic impurities

The MIC for synthetic impurities, ammonium, limonene, trimethylsilanol, and H_2S, were determined both under autotrophic (CO_2 as the carbons source) and heterotrophic (fructose as the carbon source) conditions. Heterotrophic conditions were included as a reference to assess whether any observed inhibition was associated with autotrophic metabolism or common to both autotrophy and heterotrophy. For *C. necator*, the MIC of ammonium and limonene were the same under heterotrophic and autotrophic growth conditions, whereas for trimethylsilanol and Na_2S slightly lower during autotrophic growth (Table 1). For *X. autotrophicus*, MIC for ammonium and limonene were likewise the same under heterotrophic and autotrophic conditions, although lower than for *C. necator*. Surprisingly, tolerance of *X. autotrophicus* to Na_2S did not increase under heterotrophic conditions and MIC of trimethylsilanol was higher in autotrophy compared to heterotrophy. Although growth was observed below the MIC, it was usually associated with longer lag phases than in the cultivations of the positive controls (see supplementary materials).

While only a few studies have evaluated the MIC of the compounds examined here for *C. necator* strains, no studies were found for *X. autotrophicus*. The inhibitory concentration of ammonium for *C. necator* strain DSM 531 was previously determined to be 19 g/L $(NH_4)_2SO_4$ under autotrophic conditions (Yang et al., 2021). In the current study, *C. necator* NCIMB 11599 grew still well at 26 g/L $(NH_4)_2SO_4$. Yang et al. also reported that inoculum age influences tolerance to high ammonia concentrations, with younger cultures being better adapted. In the current study, an inoculum from the early exponential phase (around OD_{600} of 0.5) was used, which might have resulted in the higher MIC, assuming no major differences between the strains.

Ammonia concentrations in raw biogas can increase to around 100 ppm when nitrogen-rich feedstocks are used (Werkneh, 2022). Ammonia is highly soluble in aqueous solutions at near neutral pH. If complete dissolution is assumed, reaching the ammonium MIC determined for *C. necator* and *X. autotrophicus* would require a long cultivation, spanning several years at a 1:1 gas-flow-to-culture-volume ratio (1 L min^{-1} per 1 L culture). Therefore, it is highly unlikely that using biogas-derived CO_2 streams as feedstock would lead to accumulation of inhibitory concentrations of ammonia for either organism in typical

Table 1
Minimum inhibitory concentrations (MIC) of impurities typically found in biogas for *C. necator* and *X. autotrophicus* 7C under heterotrophic and autotrophic growth conditions. MIC were determined after 48 h of cultivation, defined as OD_{600} not exceeding 0.3. Concentrations are given per liquid volume.

	Compound (g/L)	$(NH_4)_2SO_4$	Limonene	Trimethylsilanol	Na_2S
C. necator	Heterotrophic	52.1	40.0	4.2	>0.32
	Autotrophic	52.1	40.0	2.5	0.16
X. autotrophicus	Heterotrophic	26.0	5.0	4.2	0.08
	Autotrophic	26.0	5.0	8.3	0.08

bioreactor cultivations. Furthermore, at the concentrations commonly present in biogas, ammonia would most likely not pose a problem even during longer industrial-scale cultivations. Ammonium also serves as a nitrogen source for these bacteria and is consumed during growth, reducing its accumulation in the medium.

A MIC of 17 g/L for limonene against the food-borne pathogen *Listeria monocytogenes* has been determined previously (Han et al., 2020). Limonene inhibition has also been reported for *C. necator* H16, although the limit for complete growth inhibition was not determined (Guzman Lagunes and Winterburn, 2016). Nevertheless, a decrease in PHB content and yield was observed at 17 g/L of limonene under heterotrophic conditions. A similar trend was detected in the present study: the lag phase lasted one day in cultures without limonene, whereas exposure to 20 g/L limonene extended the lag phase to two days (see supplementary materials). Given the low aqueous solubility of limonene (14 mg/L at 25 °C; Massaldi and King, 2002), both these concentrations and the MIC determined for *C. necator* and *X. autotrophicus* exceed the saturation limit. At such concentrations, limonene is therefore separated into its own phase and may act as a solvent for cell membrane lipids, potentially impairing growth. However, further investigation is required to determine whether limonene can accumulate as a distinct layer within a bioreactor or is removed via the off-gas stream.

Trimethylsilanonol has relatively high solubility in water (1 g/L, 24 °C) compared to other organic silicon compounds (European Chemicals Agency (ECHA), 2025). Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* has been reported at MIC of approximately 20 g/L, which exceeds the saturation limit (Kim et al., 2006). Similarly, the MIC (>2 g/L) determined for *C. necator* and *X. autotrophicus* in the present study also goes above the aqueous solubility of trimethylsilanol, suggesting that inhibition may be associated with solvent effects, as observed for limonene.

H_2S inhibits hydrogenase activity (Vincent et al., 2006) and can therefore impair autotrophic growth in HOB, where all energy is derived from H_2 oxidation. This is consistent with the lower MIC observed under autotrophic conditions compared to heterotrophic conditions for *C. necator*. Consequently, maintaining H_2S concentrations in the gas feed below inhibitory threshold is essential. The comparatively higher tolerance observed under heterotrophic conditions for *C. necator* may be attributed to the presence of sulfide dehydrogenases (Lü et al., 2017). Although H_2S can undergo spontaneous oxidation readily in solutions in the presence of oxygen (Olson, 2012), which would help with HOB cultivations, this process alone did not sufficiently enhance tolerance in *X. autotrophicus* to the level of *C. necator* in heterotrophy. According to a previous approximation, the MIC of 80 mg/L determined under autotrophic conditions for *X. autotrophicus* would correspond to over 10 000 ppm of H_2S in the gas phase at pH 5.8 (pH 6.8 was used in the current work) (Xu et al., 2020). Comparable tolerance has been reported for mixed HOB cultures, with no clear inhibition observed up to several thousand ppm H_2S (Pelagalli et al., 2024). In biogas from food processing wastes, H_2S levels are typically far lower, below 1 000 ppm. With some municipal sources levels exceeding 10 000 ppm are possible (Green, 2010). However, as H_2S is corrosive to equipment, it is generally removed at an early stage of the biogas upgrading process, in which case it would not pose a problem for gas fermentation of HOB.

The relatively high MIC observed for both HOB suggest that biogenic AD and AF gas streams containing the impurities may be used as feedstocks for cultivation without extensive purification. This opens opportunities for integrating HOB-based bioprocesses directly at biowaste treating sites, where CO_2-rich off-gas could be converted to PHB. Such integration could reduce PHB production costs compared to using purified CO_2. Alternatively, as traditional biogas upgrading is a costly, unrefined biogas could be fed directly to HOB for PHB production from CO_2, while simultaneously increasing the relative methane content, as has been suggested previously (Serna-García et al., 2024). This approach could lower the cost of PHB production and create additional value to the biogas plants.

3.2. Bacteriophage enrichment from biogas digestate

Because phage contamination would be highly detrimental to large-scale production (Kamiński and Paczesny, 2024), it was investigated whether digestate from biogas plants might contain phages targeting *C. necator* and *X. autotrophicus* 7C. No bacteriophages infecting *C. necator* or *X. autotrophicus* were detected in any of the three biogas plant digestates using the soft agar overlay method applied in this study. The *Pseudomonas* sp. bacteriophage Phi6 positive control was successfully enriched from an initial concentration of $1x10^2$ PFU/ml to a minimum of $1x10^7$ PFU/ml in the presence all three digestates, confirming the functionality and sensitivity of the assay.

While bacteriophages are primarily a concern when digestate from AD or AF is used as a source of organic carbon for HOB in mixotrophic or heterotrophic cultivations, their presence in the gas phase cannot be excluded. If CO_2 from biogas plants is used as a feedstock for HOB, and bacteriophages from the digestate could enter the gas phase, there is a theoretical risk of infection. This could have catastrophic consequences, particularly when only a single production strain can be used. For instance, food regulatory approval for production of bacterial protein is strain specific.

To date, no bacteriophages have been reported for either of the bacteria used; therefore, phage enrichment from digestate was attempted. Although no active phages were detected in this study, their existence in general cannot be ruled out. As a precaution, UV treatment of gas lines carrying biogenic CO_2 could be considered, even though prolonged phage survival in the gas phase is likely limited. Survival can also depend on whether raw biogas is used or whether gas upgrading methods are applied.

3.3. Bioreactor cultivations using CO_2 from biogas plant off-gas

Limonene (21 ppm) and N_2O (16 ppm) were found to be the primary impurities in the CO_2-rich off-gas stream from biogas upgrading (see supplementary material). As H_2S corrodes equipment, it is often removed early during biogas upgrading and therefore the biogas-derived CO_2 did not contain it. D5 siloxane, CO, NO, NO_2, NH_3, HCl, HF, formaldehyde, and methanol were all under detection limit. All quantified concentrations of VOCs were under 0.1 ppm, making them unlikely to inhibit growth or function as significant carbon sources. However, given the limitations in comprehensively analyzing all trace components of the gas, bioreactor cultivations were conducted using both biogas-derived CO_2 and high-purity CO_2 (>99.5 %) for comparison. Both gases were supplemented with H_2 and O_2.

Biogas-derived CO_2 did not significantly inhibit the growth of

C. necator or *X. autotrophicus* based on the OD_{600} measurements ($p > 0.1$) (Fig. 1). Comparable results were observed by CDW measurements. Similar results have been obtained for *C. necator* cultivated on raw biogas (Serna-García et al., 2024) and for PHB production from biogas-derived CO_2 by glucose-grown cell mass of *C. necator* (Garcia-Gonzalez and De Wever, 2017). To current knowledge, this is the first study to investigate the growth of *X. autotrophicus* on biogas-derived CO_2.

With biogas-derived CO_2, slightly more biomass appeared to accumulate toward the end of the cultivations of *C. necator* (day 5, $p = 0.03$). This could be attributed either to uncharacterized organic compounds in the gas that *C. necator* can use as a carbon source, or to increased PHB production induced by some component present in the impure gas. The slime-free mutant *X. autotrophicus* SF was selected for bioreactor cultivations because preliminary experiments with *X. autotrophicus* 7C resulted in excessive foam formation, presumably due to exopolysaccharide production. However, both *X. autotrophicus* strains attached to the reactor walls when cultivation proceeded, reducing the biomass content in the broth at the end stages of the cultivations, as reflected in the OD_{600} and CDW measurements from the slime-free *X. autotrophicus* variant cultivation (Fig. 1).

PHB and protein contents were quantified at the end of the cultivations. PHB levels were similar between the two gases ($p > 0.05$) (Table 2), demonstrating that this biogenic CO_2 is a suitable carbon source for biosynthesis for these materials by *C. necator* and *X. autotrophicus*. Protein contents of *X. autotrophicus* cultures did not differ significantly between conditions ($p = 0.79$), whereas in *C. necator*, protein content was slightly lower on biogenic CO_2 compared to pure CO_2 ($p = 0.02$). Ammonium concentrations in the culture supernatants were also measured, revealing complete nitrogen consumption. This resulted in nitrogen-limitation, promoting PHB accumulation. Dissolved oxygen plateaued at 30–40 % of maximum saturation after approximately 0.5 d and 1.5 d of cultivation for *C. necator* and *X. autotrophicus*, respectively (see supplementary materials). The relatively low and stable DO levels suggest that oxygen limitation may have occurred and therefore cannot be ruled out as a possible additional trigger for PHB accumulation.

Although the impurity concentrations in the biogenic CO_2 samples were present at ppm levels in the gas phase while MIC were in g/L range in the liquid phase, continuous gas sparging may result in the accumulation of these compounds in the medium. This effect may be further enhanced in bioreactor systems equipped with gas refluxers, which are commonly used to reduce liquid evaporation and could promote entrapment of some volatile compounds in the bioreactor. Moreover, the use of raw biogas as a feedstock for HOB cultivation represents a scenario in which impurities could be present at higher concentrations. It also cannot be ruled out that impurities may show synergistic effects even at lower concentrations. In the future, prolonged cultivations could be conducted with the biogenic CO_2 streams, to attain results with potentially higher impurity accumulation.

Table 2
Biomass composition of autotrophically grown *C. necator* and *X. autotrophicus* SF using pure CO_2 and biogenic CO_2 (CO_2 derived from biogas). Cell dry weight (CDW), polyhydroxybutyrate (PHB), and protein content (estimation based on elemental nitrogen) were measured after five days of bioreactor cultivation. Values represent average and standard deviations from three biological replicates.

	Gas	CDW (g/L)	PHB (g/L)	PHB (%CDW)	Protein (%CDW)
C. necator	Pure CO_2	6.1 ± 0.1	4.7 ± 0.2	77.5 ± 3.1	20.3 ± 0.4
	Biogenic CO_2	6.7 ± 0.2	5.2 ± 0.2	77.0 ± 2.1	18.5 ± 0.6
X. autotrophicus	Pure CO_2	1.6 ± 0.1	0.5 ± 0.1	31.0 ± 4.8	45.2 ± 4.8
	Biogenic CO_2	1.4 ± 0.2	0.4 ± 0.1	28.0 ± 2.5	48.7 ± 3.9

These results suggest that biogas-derived CO_2 would be a promising feedstock for HOB-based bioproduction. Compared with CO_2 sources originating from fossil-based energy generation and industrial processes, biogenic CO_2 represents a more sustainable carbon source. While single-cell protein production from CO_2 using HOB has already reached industrial scale (Fasihi et al., 2025), PHA production using HOB is still performed using heterotrophic feedstocks, including sugars and fatty acids from sources such as corn starch, sugarcane, and vegetable oils (Zytner et al., 2013). The use of CO_2 as the primary carbon source for bioplastic production would decouple it from agriculture.

Although biogenic CO_2 streams are more sustainable than fossil-based, their cost-competitiveness is not guaranteed. The estimated capture cost for CO_2 from natural gas, biogas, and DAC ranges between 15–25, 25–90, and 250–600 € per metric ton of CO_2, respectively (Decker, 2025). These estimates indicate that biogas-derived CO_2 is currently more cost-competitive than DAC. Direct comparison of market prices is more complex, because they include additional costs related to purification, liquefaction, and transport. Future market dynamics, policy incentives, and demand for renewable CO_2 will determine whether biogenic CO_2 can become cost-competitive with fossil-derived CO_2. A comprehensive techno-economic assessment comparing biogas-derived CO_2 with alternative sources would therefore be valuable.

3.4. Acid fermentation

AF was conducted to produce CO_2 and H_2 for HOB cultivations. From both experiments, BW1 (S/I 1:1) and BW2 (S/I 2:1), the main component of produced gas was CO_2 (data not shown). Main differences

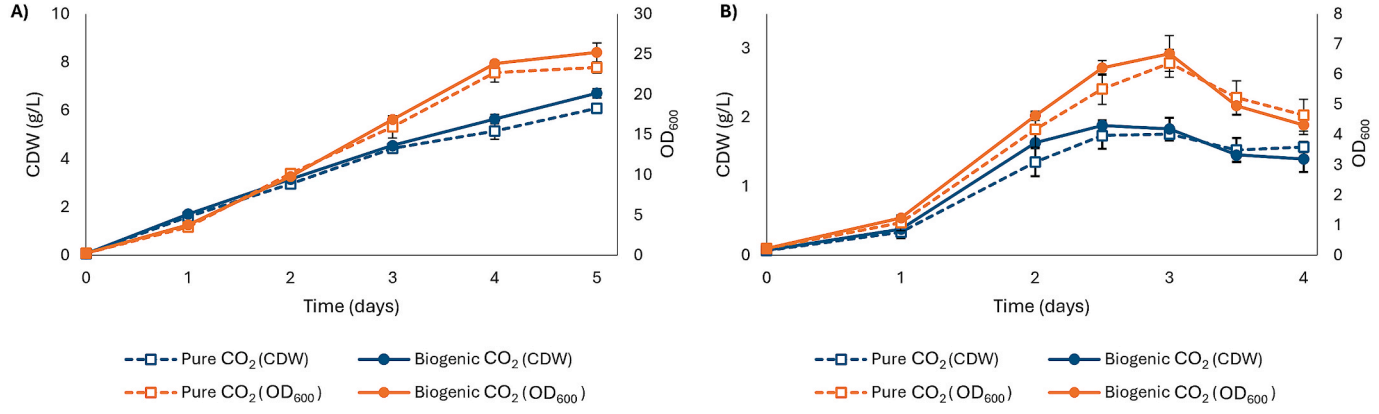

Fig. 1. Autotrophic growth of A) *C. necator* and B) *X. autotrophicus* SF on pure CO_2 and biogenic CO_2 (CO_2 derived from biogas). Data represents the average from three biological replicates, with error bars indicating the standard deviations.

between BW1 and BW2 were seen in H_2 and CH_4 concentrations. Higher organic loading in AF (BW2) resulted in higher H_2 yields and slightly higher VFA concentrations (see supplementary material) but lower gas volumes (Fig. 2). With lower loading (BW1), the methanogen deactivation was not completely successful as the produced gas contained CH_4 in higher concentrations than in BW2. In addition, the gas production rate differed between the trials (see supplementary material); the gas production was higher with BW2 for the first 100 h of the experiment but ultimately resulted in higher volumes with BW1, most likely due to the production of CH_4 towards the end of the experiment.

The pH measured at the end of the experiment (Fig. 2) was decreased below 6 in BW2, which is a sign of acidification, whereas in BW1 the final measured pH of around 7 indicates less significant acidification compared to the beginning of the experiment (7.8–8.0 and 7.6–7.8 for BW1 and BW2, respectively). In fermentation experiments targeting H_2 production with a variety of organic substrates, lower pH, especially 5.0–6.0, is often regarded optimal in terms of methanogen inhibition and simultaneous boost of H_2-producing bacteria, which in turn can increase both the H_2 production rate and yield (Alexandropoulou et al., 2018; Chu et al., 2008; Wainaina et al., 2019).

Only a few studies have reported the composition of produced gases from AF, with most targeting either H_2 or VFA production. Lauri et al. (2021) reported a gas composition of 85 % CO_2, 12 % H_2, and 2 % CH_4 in a continuous AF of mixed composition of food waste and sewage sludge at pH 5.0–5.5. These values align well with the composition of the gas produced in BW2 in the present study, especially regarding H_2 (Table 3). As in the current work, in the study by Lauri et al. (2021), the pH was left uncontrolled, and the buffering impact of the substrate-inoculum mixture kept the pH rather low and favorable for the active microbial consortia. Moreover, the reported organic loading rate for this specific result was rather high, 10–13 kg VS/(m^3·d), targeting a similar acidification impact by organic overloading as in the present study, expressed as S/I ratio (VS-based) of 2:1 for BW2. Overall, reported AF gas compositions vary substantially, and process conditions are often not fully reported. Therefore, determining the gas composition obtained from AF requires more research to produce suitable gas mixtures for gas fermentation.

In addition to the cost and availability of CO_2, the economics of HOB-based bioproduction are strongly influenced by the supply of green H_2. When H_2 is produced via water electrolysis, the cost of renewable electricity is one of the main contributors to HOB production costs (Fasihi et al., 2025). Therefore, alternative ways of producing H_2, such as acid fermentation, may improve process economics. However, the benefits of AF should be evaluated at the system level. Specifically, it is essential to evaluate whether maximizing direct energy recovery (CH_4 production via AD) or producing higher-value chemical intermediates (VFAs via AF) provides greater overall benefit. This decision depends not only on energy efficiency, but also on downstream product value, process stability, and compatibility with existing infrastructure.

Because AF diverts carbon toward VFAs and H_2 rather than CH_4, a significant fraction of the substrate energy remains in VFA intermediates. These compounds could serve as co-substrates in mixotrophic PHA production by HOB, where CO_2 and VFAs are utilized simultaneously. Some VFAs, such as valeric and propionic acids, can provide monomers other than 3-hydroxybutyrate, potentially increasing polymer flexibility (Jawed et al., 2022). As a result, integrating AF with mixotrophic PHA production may represent an economically attractive strategy for upgrading biowaste.

3.5. *Growth experiments using gas from acid fermentation*

CO was identified as the most substantial impurity in the gas produced by AF (Table 3), and its concentration was observed to increase with higher organic loading (BW2). Similarly, an increase was also noticed for limonene and N_2O. Due to the limited volume of gas produced, it was only possible to quantify impurities using FTIR and GC. Nevertheless, the produced gases likely contained H_2S since, unlike in biogas upgrading process, there was no H_2S removal step. For instance, Lauri et al. (2021) reported a H_2S concentration of 0.04 % in a similar batch-mode experiment.

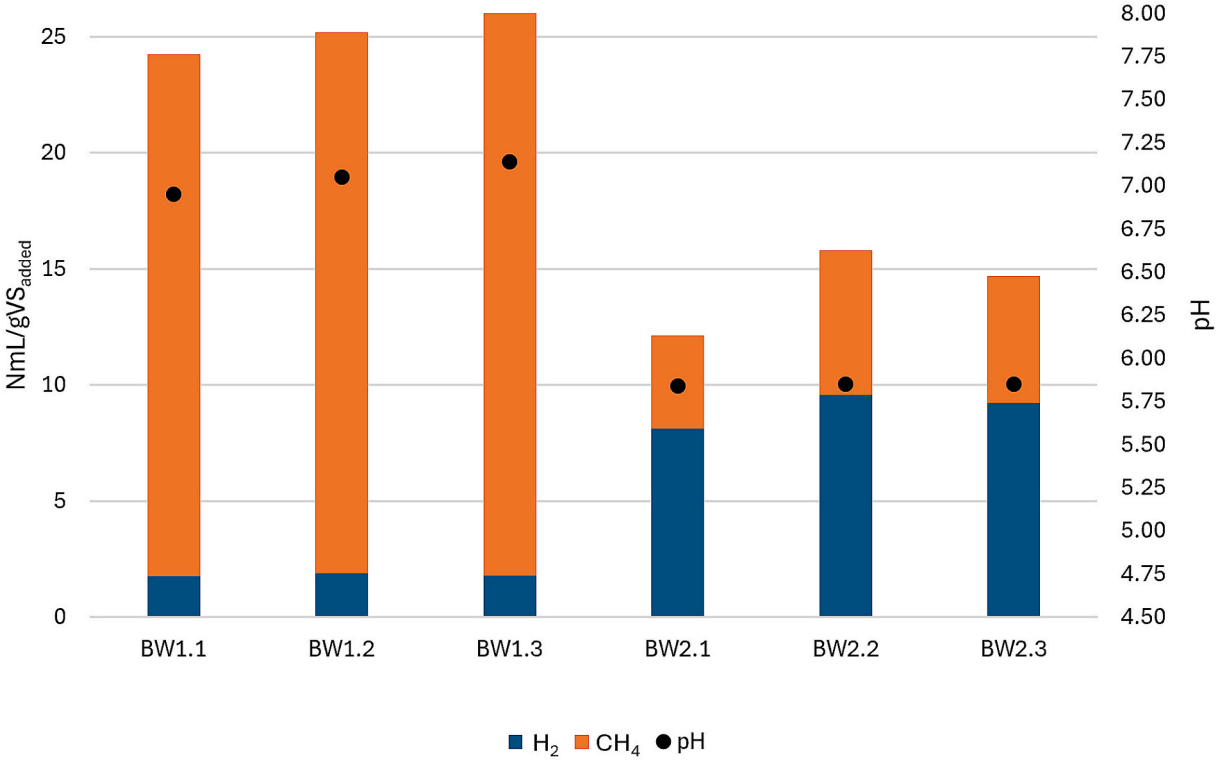

Fig. 2. H_2 and CH_4 production (NmL/gVS$_{added}$) and final pH measured from AF experiments BW1 and BW2 with three technical replicates.

Table 3
Gas compositions of the acid fermentation (AF) gases (BW1 and BW2 measured) and gas mixtures used for test tube cultivations of *C. necator*. AF gases were supplemented with H_2 and O_2 (G1 and G2), and simulated gas mixtures (G1 and G2 simulated) with pure gases were used as controls for cultivating *C. necator*. Concentrations are expressed at wet NTP conditions (101.3 kPa, 0 °C).

Component	BW1 measured	G1	Simulated G1	BW2 measured	G2	Simulated G2
CO_2, vol-%	68.9	10.0	10.0	79.3	10.0	10.0
H_2, vol-%	1.7	76.2	76.0	12.0	79.2	77.7
O_2, vol-%	2.6	9.9	9.5	1.8	9.9	9.7
CH_4, vol-%	19.6	2.8		5.5	0.7	
N_2, vol-%	6.7	1.0	4.5	4.6	0.6	2.6
H_2O, vol-%	0.8	0.1		1.1	0.1	
CO, ppm	44.0	6.3		117.0	14.9	
N_2O, ppm	< 0.1			2.1	0.3	
Limonene, ppm	< 2.0			10	1.3	
NH_3, ppm	2.0	0.3		< 1.0		

AF gases were supplemented with H_2 and O_2 to achieve optimal gas ratios for HOB growth, resulting in gas mixtures G1 and G2 (Table 3). For comparison, simulated gas mixtures of pure gases with similar compositions were prepared as positive controls (simulated G1 and G2) for the test tube cultivations.

The growth of *C. necator* on AF-derived CO_2 and pure CO_2 was comparable during exponential phase (Fig. 3). After the cultivations reached stationary phase, the headspaces were replaced with fresh gases, and the cultivations were continued. For technical reasons, there was a 57-h gap between the first cultivation and the second one, during which the cultures were stored mostly on ice, except while changing the gas phases of the headspaces. Similar growth during the first stage suggests comparable metabolic states for the cells before the 57 h storage on ice, which likely affected both conditions similarly. In all

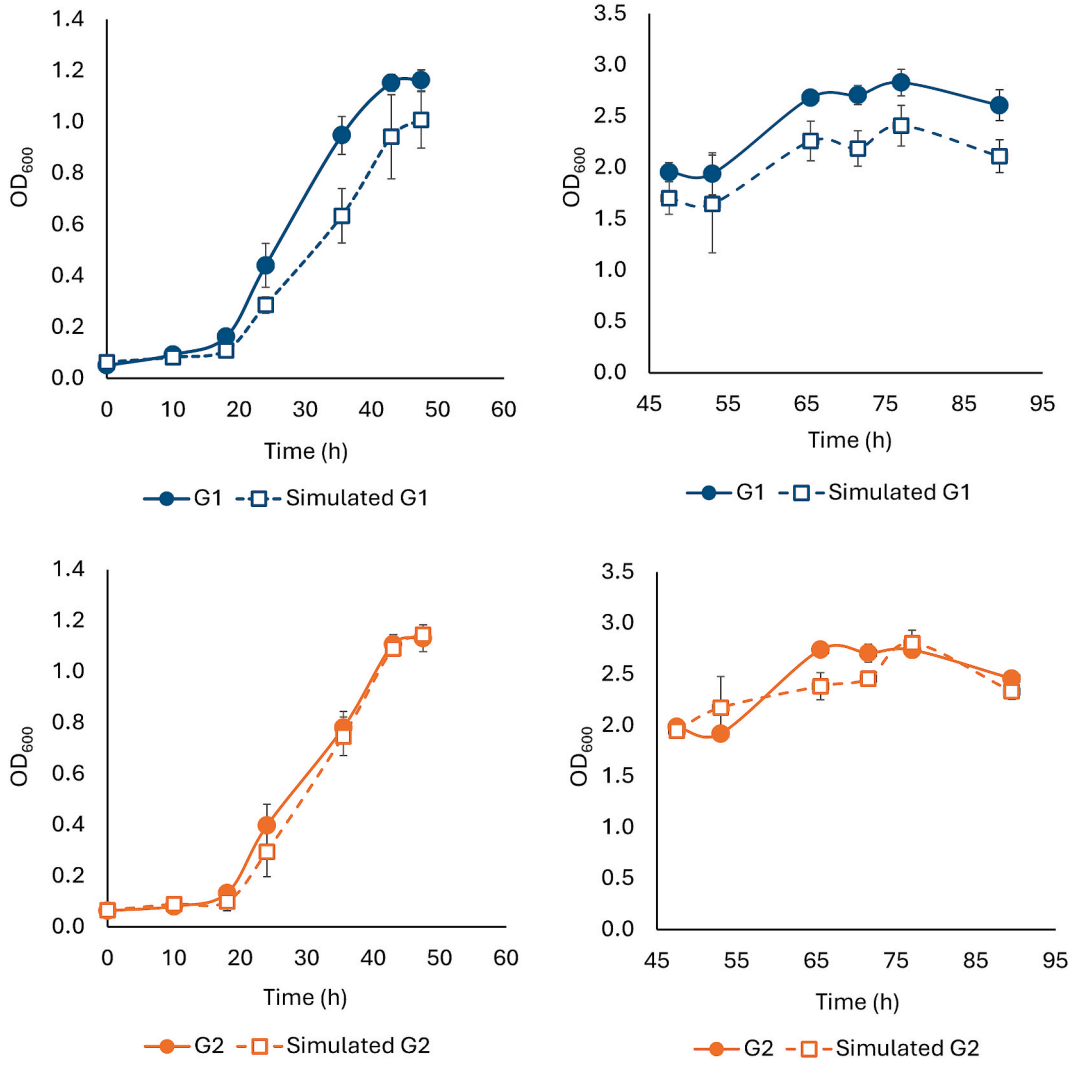

Fig. 3. Growth of *C. necator* on acid fermentation gases supplemented with H_2 and O_2 (G1 and G2) and pure gases (simulated G1 and G2) with similar gas compositions. The compositions of the gas mixtures, all containing 10 % CO_2, are presented in Table 3. The growth curves on the right were determined after replacing the gas phases with fresh ones.

cultivations, the OD_{600} increased (0.7–0.8 OD_{600} units) during the period on ice, indicating that significant growth had taken place. *C. necator* produces PHB as a storage material for carbon under nutrient limitation. Possibly O_2 was limiting at the end of the first cultivation phase and the PHB was rapidly consumed when O_2 was again provided during replenishing the gases. Growth in the second cultivation phase was modest. Nonetheless, the test tube cultivations under the gases demonstrated that AF-derived gases did not inhibit the growth of *C. necator* and could serve as the source of sole carbon (Fig. 3). In fact, the end point OD_{600} of G1 was somewhat higher than that of the simulated G1 ($p = 0.02$). No comparable effect was observed for G2 gas.

An advantage of AF over AD is the simultaneous production of CO_2, H_2, and VFA, all of which can serve as substrates for HOB. Cultivation on AF-derived CO_2 and H_2 demonstrated in this study appears promising, as *C. necator* grew in comparable manner to cultures supplied with pure gases. However, the gas volumes applied in test tubes were rather small considering potential impurity accumulation. Therefore, cultivations should be performed at a larger scale with sparging of AF-derived gases in bioreactors to better assess possible inhibitory effects. In a previous study, CO_2 and H_2 generated via AF of melon mono-waste were used to produce 1.7 g/L PHB by *C. necator* DSM545 (Costa et al., 2023). However, pure CO_2 was not used as a control, and impurities present in the biogenic CO_2 stream were not characterized. Nevertheless, taken together, the results of that study and the present work support the potential of AF as a source of gases for HOB cultivation without the need for purification. In the future, techno-economic assessment is needed to evaluate whether AF could represent a viable alternative to conventional AD.

4. Conclusion

HOB require concentrated CO_2 sources for efficient autotrophic growth. In this study, CO_2 derived from AD and AF supported the growth of *C. necator* and *X. autotrophicus* at levels comparable to pure CO_2. The high tolerance of both strains to typical biogenic gas impurities suggests that extensive CO_2 purification may not be required. While AD provides concentrated CO_2, AF offers the additional advantage of generating H_2 and VFAs alongside CO_2, creating mixotrophic production opportunities. Overall, these findings demonstrate the feasibility of using biogenic CO_2 sources as sustainable feedstocks for autotrophic cultivation of HOB-based carbon capture and bioproduction.

CRediT authorship contribution statement

Tytti Jämsä: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Milla Tynkkynen:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Markku Vainio:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Tuula Kajolinna:** Writing – review & editing, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Saija Rasi:** Writing – original draft, Funding acquisition, Formal analysis, Conceptualization. **Antti Nyyssölä:** Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2026.135357.

Data availability

Supplementary materials of this work can be found in the online version of the paper. Data will be made available on request.

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