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Establishing Mixotrophic Growth of *Cupriavidus necator* H16 on CO_2 and Volatile Fatty Acids

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Abstract: The facultative chemolithoautotroph *Cupriavidus necator* H16 is able to grow aerobically either with organic substrates or H_2 and CO_2 s and it can accumulate large amounts of (up to 90%) poly (3-hydroxybutyrate), a polyhydroxyalkanoate (PHA) biopolymer. The ability of this organism to co-utilize volatile fatty acids (VFAs) and CO_2 as sources of carbon under mixotrophic growth conditions was investigated and PHA production was monitored. PHA accumulation was assessed under aerobic conditions, with either individual VFAs or in mixtures, under three different conditions—with CO_2 as additional carbon source, without CO_2 and with CO_2 and H_2 as additional sources of carbon and energy. VFAs utilisation rates were slower in the presence of CO_2. PHA production was significantly higher when cultures were grown mixotrophically and with H_2 as an additional energy source compared to heterotrophic or mixotrophic growth conditions, without H_2. Furthermore, a two-step VFA feeding regime was found to be the most effective method for PHA accumulation. It was used for PHA production mixotrophically using CO_2, H_2 and VFA mixture derived from an anaerobic digestor (AD). The data obtained demonstrated that process parameters need to be carefully monitored to avoid VFA toxicity and low product accumulation.

Keywords: mixotrophic fermentation; polyhydroxyalkanoates; anaerobic digestion; volatile fatty acids

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1. Introduction

Up to 348 million tons of plastics are produced each year, a figure significantly higher than the weight of the human adult population [1,2]. An estimated 99 million Mt of mismanaged plastic waste (MPW) was generated in 2015 [3]. MPW is regarded as plastic waste that has been littered, inadequately disposed or released from uncontrolled landfill sites [4]. Between 1950 and 2015, only 9% of plastic waste generated was recycled, while 12% were incinerated, with almost 80% left to go to landfills and the natural environment [5]. Although there has been a huge effort to recycle plastics, current rates are still low at 14% to 18% with the EU topping the chart at 30% [5]. This has led to significant pollution of aquatic and terrestrial habitats, owing to their recalcitrant nature, causing significant damage to the environment, human health, coastal life, amongst others [3]. The public health and environmental impact of plastic pollution, coupled with the constantly depleting fossil resource for chemo-synthetic plastic manufacturing, has necessitated the need

for alternative sources of plastics. Polyhydroxyalkanoates (PHA) are bio-based thermoplastics produced by several organisms as carbon and energy storage material, in the presence of abundant carbon and a limiting nutrient such as oxygen or nitrogen [6]. PHA have properties similar to their synthetic counterparts, with the added advantage of biodegradability, biocompatibility and they can be synthesised from renewable carbon sources. However, they are more expensive to produce than chemo-synthetic plastic, thus making them less economically attractive.

Cupriavidus necator also known as *Ralstonia eutropha* or *Alcaligenes eutrophus* is a well-studied Gram-negative β-proteobacteria, capable of autotrophic growth, with CO_2 as the sole carbon source, H_2 as electron donor and O_2 as electron acceptor [7–9]. It can accumulate up to 12 PHA granules per cell, amounting to around 90% of its cell dry weight [10,11]. Its ability to oxidize hydrogen and fix CO_2 via the Calvin-Benson-Basham (CBB) cycle, make it a suitable candidate for economically viable PHA production. Utilising CO_2 for PHA production has double advantages. First, it can help drive down the price of PHA, owing to its abundance and renewable source, thus making it an attractive alternative to fossil-based plastics. Secondly, it presents a route to a carbon neutral or net-zero-CO_2 world, thus contributing to achieving the objectives of the Paris Agreement to limit climate change [12].

Consequently, several studies have reported the use of *C. necator* as a microbial cell factory to produce PHA from CO_2 [13–15]. However, these studies have mainly focused on optimising polyhydroxybutyrate (PHB) synthesis, a homopolymer of PHA that is produced predominantly by *C. necator*. Although PHB has unique properties including biodegradability and synthesis from renewable sources, it is very brittle and stiff with high crystallinity (>60%). These properties make them unsuitable for many applications. Incorporating other monomers of hydroxyalkanoates (HA) into the polymer's structure does improve the property of the polymer. For example, polyhydroxyvalerate (PHV) is more flexible, with lower crystallinity, compared to PHB due to the possession of an ethyl group in its structure in place of the methyl group in PHB. Thus, incorporating HV monomeric units either randomly or in blocks into the polymer structure to form polyhydroxybutyrate-co-hydroxyvalerate (PHBV) results in polymers with properties that are more suited to a wider range of applications compared to PHB [6].

To date, most research aimed at producing co-polymers of PHA has relied solely on organic substrates such as fatty acids, triglycerides, organic waste, carbohydrates, or sugars, as carbon sources [16,17]. Very few studies have investigated the production of PHA copolymers under mixotrophic (autotrophic and heterotrophic) growth conditions [18,19]. In both examples, co-polymers were produced from pure precursor substrates, and in one case [19], they were produced using biomass grown on sugars. While the results of these studies clearly show the potential of mixotrophic co-polymer production by *C. necator*, the use of pure fatty acids as precursor substrates or biomass from sugars does significantly increase the overall production cost of the polymer, reducing its economic competitiveness compared with synthetic plastics.

Anaerobic digestion (AD) is a process by which organic waste of domestic, industrial, or agricultural origins is broken down in the presence of a complex microbial community. This process generates large quantities of both CO_2 and VFAs as by-products. Using this dual waste-stream as carbon and additional energy source, without the need for recovery and separation of VFAs from anaerobic digestates has major technical and economic advantages, thereby reducing overall capital cost [9,20]. Additionally, the presence of volatile acids such as propionate or valerate in the AD would result in copolymers with improved properties. Consequently, direct utilisation of AD-derived VFAs and CO_2 for the mixotrophic production of PHAs presents an exciting opportunity to produce copolymers with desirable physicochemical properties whilst providing efficient carbon recovery and circularity.

Thus, in this study, we explored the potential of *C. necator* H16 to produce PHA under mixotrophic conditions, using AD derived VFAs and CO_2 as cheap and renewable carbon

sources. Initial experiments focused on process optimisation using synthetic VFAs as additional carbon and energy source while supplying CO_2 and H_2 into the reactor. As VFAs have shown toxicity in a wide range of Gram-negative organisms [21–24], the toxic effect of VFAs was also investigated. Due to their inherent toxicity and inhibitory effect, a two-step fermentation process was found to be the best strategy to achieve mixotrophy and this strategy was used for PHA production from CO_2 and AD derived VFAs.

2. Materials and Methods

2.1. Microorganism and Growth Condition

C. necator H16 (DSM 428) purchased from the DSMZ strain collection (Braunschweig, Germany) and preserved in cryo-beads at −80 °C, was used in this study. Cells were recovered on Luria Broth (LB) plates and grown at 30 °C. For fermentation experiments, starter cultures were prepared by inoculating 5 mL of LB with a single colony, incubated at 30 °C at 200 rpm for 24 h. PHA production was carried out in a chemically defined minimal medium (MM) [25,26] having modified SL7 trace element solution [27] and supplemented with synthetic or natural AD derived VFAs.

For heterotrophic growth, cells were grown in 250 mL shake flasks containing 50 mL minimal medium with 0.5 g/L NH_4Cl and supplemented with individual VFAs (50 mM carbon equivalent i.e., 50 mM formate, 25 mM acetate, 16.67 mM propionate, 12.5 mM butyrate, 10 mM valerate and 8.33 mM hexanoate). Samples were collected at regular intervals for growth, PHA quantification and VFA composition analysis.

2.2. Substrate Utilisation and Toxicity of VFAs

Toxicity assay was performed in a BioLector system (m2p-labs, Baesweiler, Germany) which allows high-throughput screening of fermentation processes on a microscale. Primary cultures of *C. neactor* H16 cells were cultivated in LB medium as described above and 1% primary culture was used to inoculate 1 mL MM medium in 48 well microtiter flower plates (MTP-48, m2p Labs, Baesweiler, Germany), containing individual VFAs at different concentrations (10–50 mM). Cells were grown at 30 °C and 1200 rpm and an initial pH of 6.9. The growth was monitored online every 30 min without interruption of the process. A standard curve was plotted to correlate scattered light reading from the biolector with the OD_{600} readings of a spectrophotometer. All cultivations were performed in triplicates.

2.3. Bioreactor Studies

2.3.1. PHA Production from VFAs and from VFAs and CO_2

Batch fermentation experiments to compare PHA production with VFAs as the sole source of carbon and PHA production from VFAs and CO_2 were carried out in a 3 L New Brunswick™ BioFlo®/CelliGen® 115 Bioreactor (Eppendorf SE, Juelich, Germany) with a working volume of 1.5 L. Gasses were supplied via a Bronkhorst High-Tech EL-FLOW/IN-FLOW mass flow meters/controllers (MFCs) (Bronkhorst, Suffolk, UK). A 2-point calibration of the pH probe (Metler Toledo Ltd., Leicester, UK) was carried out at pH = 4 and pH = 7 using standard pH buffers prior to autoclaving of the vessel at 121 °C for 15 min. After autoclaving, MM with 10 mM of each VFA was aseptically filtered into the reactor through a 0.2 μm Sartopore Capsule PES Filter (Sartorius, Surrey, UK). The dissolved oxygen (DO) probe (INPRO 6000 sensor; Metler Toledo Ltd., Leicester, UK) was then calibrated under air-saturated conditions as 100%, and under nitrogen saturated conditions as 0%. Total gas flow was kept at 1 vvm consisting of 100% air or 97% air and 3% CO_2 for VFAs only or VFAs plus CO_2 experiments, respectively. The temperature was maintained at 30 °C, while pH was monitored and maintained at 6.9 with a dead band of 0.05 using 1 M HCl or NaOH. The dissolved oxygen (DO) was monitored and was kept at a minimum of 40% using an agitation cascade between 600 and 1000 rpm. Starter cultures were prepared in LB medium as described earlier, centrifuged and the biomass was

washed with PBS. After which the biomass was resuspended in fermentation MM medium, and the reactor was inoculated to a starting OD of 0.2. The reactor was run for 72 h and samples were collected regularly for OD measurements, PHA quantification and VFA utilisation measurements.

2.3.2. PHA Production from VFAs, CO_2 and H_2

PHA production from VFAs and CO_2 with H_2 as an additional energy source was performed in a 1.3 L DASGIP Parallel Bioreactor System (Eppendorf SE, Juilich, Germany). The reactor setup is as described previously [9]. Prior to vessel sterilisation at 121 °C for 15 min, the pH probe (Metler Toledo Ltd., Leicester, UK) was calibrated by a 2-point calibration at pH = 4 and pH = 7, using standard pH buffers. After sterilisation, MM with a known concentration of VFAs or anaerobic digestate (AD) was filtered into the vessel, followed by in-situ 2-point calibration of the optical DO probe (Metler Toledo Ltd., Leicester, UK) at 0% pO_2, and 100% of pO_2 by nitrogen and air saturation, respectively, at atmospheric pressure. Both CO_2, air and H_2 were supplied at a total flow of 1 vvm and percentage composition of 3%, 19% and 78%, respectively. pH was monitored and kept at 6.9 using 1 M NaOH while DO concentration was kept at a minimum of 5% using a cascaded agitation. The bioreactor off-gases were fed into an external foam trap bottle, fitted with a Visiferm Hamilton oxygen optical probe and was maintained at a maximum O_2 (*v*/*v*) concentration of 4%. Probe was calibrated at 0% (*v*/*v*) and 10% (*v*/*v*) O_2 with N_2 and N_2 with 10% (*v*/*v*) O_2, respectively. To maintain a non-flammable atmosphere in the reactor headspace, a program was written into the Dasware ® software, which in response to O_2 concentration (*v*/*v*) in the headspace exceeding 5% (*v*/*v*), replaced the gas mixture being supplied with N_2. Off-gas composition was also analysed inline using a multiplex Raman Laser Analyser (Atmosphere Recovery Inc., Eden Prairie, Minnesota, USA) positioned after the foam trap.

Starter cultures were prepared as described above and the reactor was inoculated to a starting OD of 0.2 and at a working volume of 0.75 L. Samples were taken regularly to analyse the growth, PHA concentration and VFA composition offline.

2.4. PHA Quantification Using GC-MS

The content of intracellular PHA was determined by GC-MS, using a previously described method [28] with the following modifications. Approximately 25 mg of lyophilised cells were subjected to methanolysis in Teflon capped glass vials with 2 mL of 5% sulphuric acid, 2 mL chloroform and benzoic acid added as internal standard. The vials were then incubated for 4 h at 100 °C and subsequently cooled to room temperature; 4 mL of 1 N NaCl solution in HPLC grade H_2O (Sigma Aldrich UK, Dorset, UK) was added to the vials and the mixture was vortexed for approximately 30 s. After phase separation, 1 mL of the organic phase containing methylated monomers, was transferred to GC vials and injected into an Agilent 6890 Gas chromatograph with 7673 autosampler (Agilent Technologies, Cheshire, UK), equipped with a J and W DB-Innowax column, 20 m length × 0.18 mm diameter × 0.18 μm film thickness (Agilent Technologies, Cheshire, UK) and an Agilent 5973 Mass selective detector (Agilent Technologies, Cheshire, UK). Hydrogen was used as the carrier gas at a flow rate of 0.6 mL/min. PHA contents were estimated from Standard curves of hydroxybutyrate using commercial P-3HB (Sigma Aldrich, Dorset, UK). Concentrations of Hydroxyvalerate (HV) were estimated by calculating the peak area relative to HB.

2.5. Quantification of Volatile Fatty Acids Using HPLC

For VFA quantification, culture samples were centrifuged for 15 min at 8000 rpm and the supernatant was collected. Standard solutions of each VFAs within the expected range of concentrations were prepared. The supernatants, standard solutions and blanks were filtered using a 0.2 μm syringe filter, and 0.5 mL of filtered solution was mixed with 0.5

mL of diluent solution (0.005 M H_2SO_4) to give a 1:1 *v*/*v* ratio. The mixture was thoroughly vortexed and transferred into HPLC vials with split caps. Samples were run in an isocratic mode for 55 min at 35 °C on a Dionex UltiMate 3000 HPLC system (ThermoFisher Scientific, Waltham, MA, USA), with 0.005 M H_2SO_4 used as the mobile phase and an Aminex HPX-87H 300 mm × 7.8 mm × 9 µm column (Bio-Rad, Watford, UK) used as the stationary phase. A standard curve was used to obtain VFA concentrations in samples.

3. Results

3.1. Toxicity of Volatile Fatty Acids (VFAs) to C. necator H16

Production of PHA from VFAs by *C. necator* will depend on the ability of the cells to tolerate and utilize VFAs for growth and polymer accumulation. Thus, the growth profile of *C. necator* H16 using various concentrations (0 mM to 50 mM) of individual VFAs (formic acid, acetic acid, propionic acid, butyric acid, valeric acid and hexanoic acid) was tested. We found that cells were able to utilise each VFA as the sole carbon and energy source up to a certain concentration, with an observed increase in the lag phase with increasing concentrations of VFAs and an increase in their chain length (Figure 1).

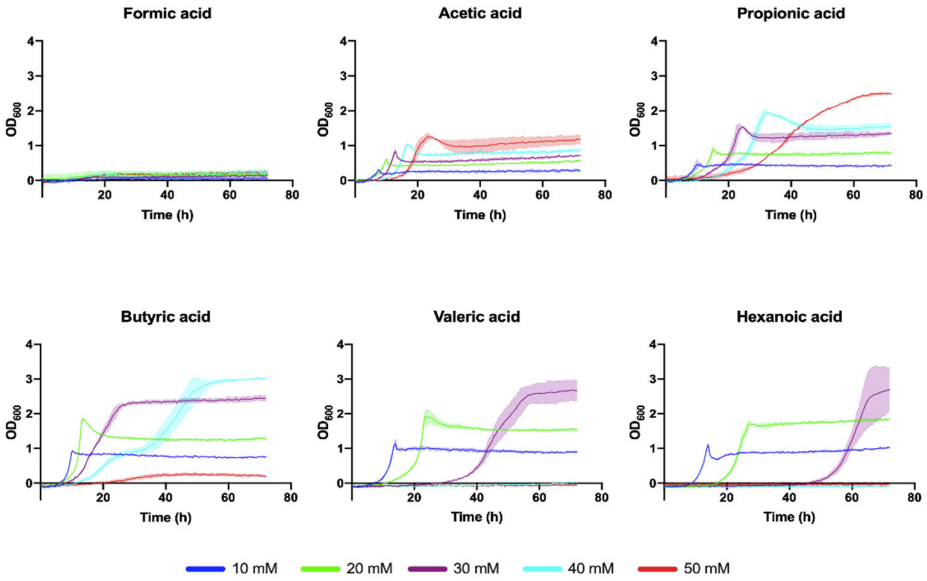


Figure 1. Growth of *C. necator* H16 with varying concentration of volatile fatty acids (VFAs). Legend: 10 mM—blue, 20 mM—green, 30 mM—purple, 40 mM—cyan, 50 mM—red.

Specifically, for valeric (C5) and hexanoic (C6) acids, concentrations above 10 mM led to a dramatic increase in lag phase (up to 50 h), with complete growth inhibition observed above 30 mM for both compounds (Figure 1). A similar trend can also be seen for butyric, propanoic and acetic acids, with lag phase increasing to 30 h, 18 h and 15 h, respectively, at 50 mM concentration. Taken together, these results indicate that at higher concentrations all VFAs show increased levels of toxicity to *C. necator* H16.

3.2. Biomass and PHA Accumulation by C. necator H16 Using Individual VFAs

C. necator H16 was grown aerobically with individual VFAs normalised to 50 mM carbon equivalent (50 mM formate, 25 mM acetate, 16.67 mM propionate, 12.5 mM butyrate, 10 mM valerate and 8.33 mM hexanoate) to compare each VFA for biomass and PHA production. We chose 50 mM carbon equivalent concentration based on the results obtained from the toxicity assay. We observed that increase in the biomass was proportional to VFAs chain length up to butyric acid, as previously observed in the toxicity study. Under heterotrophic growth, biomass accumulation was the highest when butyric acid was

used as a carbon source (cell dry weight (CDW) of 0.65 g/L). Formic, acetic, propionic, valeric and hexanoic acid had biomass concentrations of 0.1 g/L, 0.31 g/L, 0.47 g/L, 0.56 g/L and 0.63 g/L at 72 h, respectively (Table 1).

Table 1. Growth and PHA production of *C. necator* H16 at 72 h post inoculation using 50 mM carbon equivalent VFAs in shake flasks under heterotrophic conditions.

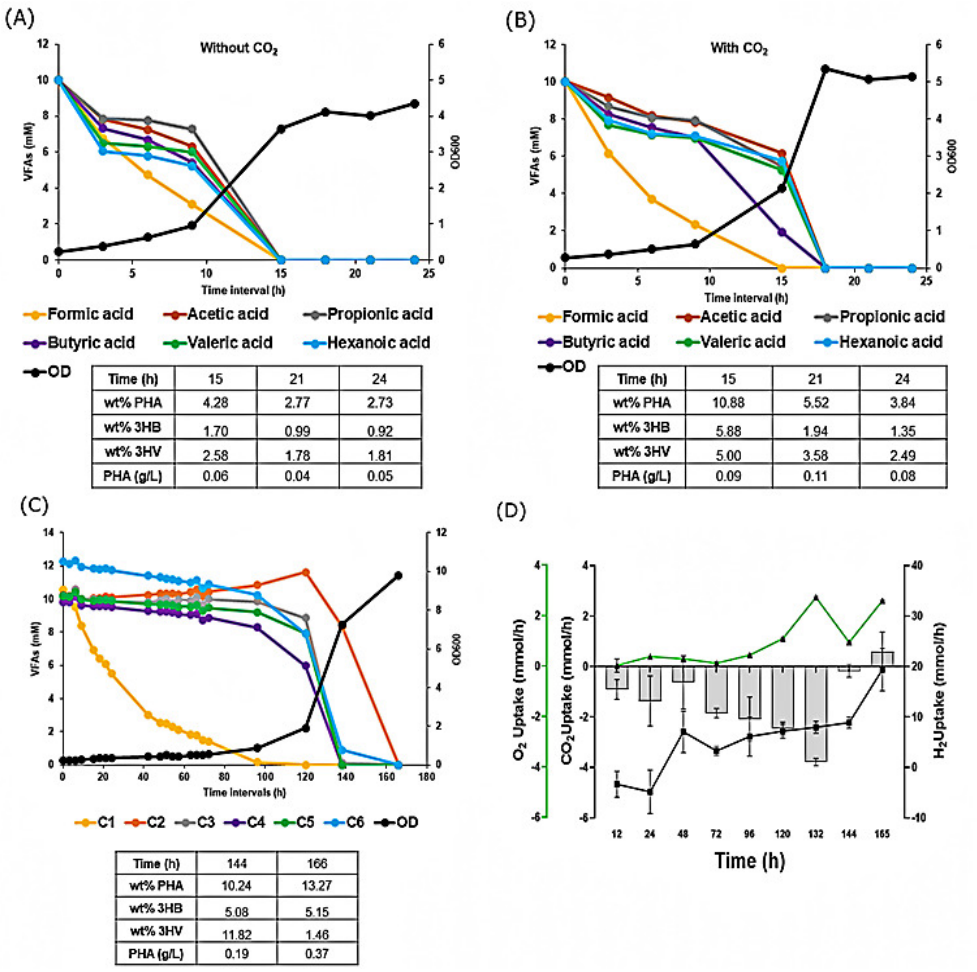
VFA (50 mM Carbon Equivalent)	CDW (g/L)	PHA (g/L)	PHA (wt% g/gCDW)	3HB (wt% g/gCDW)	3HV (wt% g/gCDW)
Formate [a]	0.10 (±0.01)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	N.D [g]
Acetate [b]	0.31 (±0.00)	0.00 (±0.00)	0.07 (±0.12)	0.07 (±0.12)	N.D
Propionate [c]	0.47 (±0.01)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	N.D
Butyrate [d]	0.65 (±0.02)	0.01 (±0.00)	0.85 (±0.05)	0.85 (±0.05)	N.D
Valerate [e]	0.56 (±0.01)	0.06 (±0.01)	10.18 (±0.92)	0.28 (±0.07)	9.90 (±0.85)
Hexanoate [f]	0.63 (±0.01)	0.00 (±0.00)	0.11 (±0.01)	0.11 (±0.01)	N.D

[a] 50 mM formate, [b] 25 mM acetate, [c] 16.67 mM propionate, [d] 12.5 mM butyrate, [e] 10 mM valerate, [f] 8.33 mM hexanoate and [g] N.D Non detected.

In terms of PHA production, very little PHA was accumulated under heterotrophic conditions. Low PHA accumulation was somewhat expected as only 50 mM carbon equivalent VFA was present in the culture medium, and this was primarily used for biomass formation. Cells grown with valeric acid had the highest percentage PHA yield per cell dry weight of 10.18 (±0.92), consisting mainly of 3HV units (Table 1). Furthermore, no PHA accumulation was observed when cells were grown in either formate or propionate (Table 1) and only 0.07 wt% (±0.12) PHA per CDW was detected in the presence of acetate.

3.3. Biomass and PHA Accumulation by C. necator H16 Using VFA Mixtures and CO_2 in Bioreactors

For mixotrophic feeding experiments, batch cultivation of *C. necator* H16 was performed in minimal medium supplemented with a mixture of C1-C6 VFAs, at a concentration of 10 mM each under three different conditions in bioreactors: (1) with CO_2 as additional carbon source, (2) without CO_2 and with (3) CO_2 and H_2 as additional sources of carbon and energy, respectively. We observed significant variation in growth and PHA profiles under these conditions (Figure 2A–C).



Time (h)	15	21	24
wt% PHA	4.28	2.77	2.73
wt% 3HB	1.70	0.99	0.92
wt% 3HV	2.58	1.78	1.81
PHA (g/L)	0.06	0.04	0.05

Time (h)	15	21	24
wt% PHA	10.88	5.52	3.84
wt% 3HB	5.88	1.94	1.35
wt% 3HV	5.00	3.58	2.49
PHA (g/L)	0.09	0.11	0.08

Time (h)	144	166
wt% PHA	10.24	13.27
wt% 3HB	5.08	5.15
wt% 3HV	11.82	1.46
PHA (g/L)	0.19	0.37

Figure 2. VFA utilisation and PHA production by *C. necator* H16 cultures grown in a bioreactor. VFA was fed to the reactor as a mixture. Panel (**A**) cultures grown with VFAs without CO_2 as an additional source of carbon; Panel (**B**) cultures grown with VFAs and with CO_2 as an additional source of carbon; Panel (**C**) cultures grown with VFAs and with CO_2 and H_2 as additional sources of carbon and energy. Panel (**D**) shows CO_2 and H_2 gas consumption by *C. necator* H16 cultures grown with CO_2 and H_2 as additional sources of carbon and energy. CO_2 uptake -—grey bars, H_2 uptake — black line and O_2 uptake -— green line. The wt% in g/gCDW of PHA and PHA yields are presented in the tables below for each growth condition, below Panels (**A**—**C**).

Firstly, *C. necator* H16 was grown with a mixture of VFAs (C1–C6, 10 mM each) in the presence or absence of CO_2, and PHA accumulation was measured at 15, 21 and 24 h of cultivation (Figure 2A and 2B). In both fermentations (Figure 2A, B), formic acid was utilised faster compared with other VFAs with higher carbon chain lengths. VFAs were utilised faster in the absence of CO_2, with all available VFAs consumed at 15 h and 18 h of fermentation in the presence and absence of CO_2, respectively, (Figure 2A, B). In the presence of CO_2, the OD and PHA content observed at 24 h were 5.14 and 3.84 wt%, respectively, higher than 4.12 and 2.73 wt% observed in the absence of CO_2. Additionally, PHA content was at least 1.5 times higher at all time points studied when CO_2 was present, and the 3HV content (per total PHA) was lower in the presence of CO_2 particularly within the first 15 h when formate was present and providing reducing equivalent for CO_2 fixation. (Figure 2B). Taken together, these results suggest mixotrophic growth.

Secondly, the same VFA mixture (C1–C6, 10 mM each) was added to the growth medium with a continuous supply of CO_2 and H_2 as additional sources of carbon and energy. Under this condition, we observed a very slow growth and VFA utilisation, with a growth

rate of 0.021 h^{-1} and an OD of 0.36 at 24 h (Figure 2C). Additionally, only after 166 h of cultivation were all the VFAs consumed. Compared to either growth from VFAs only or VFAs and CO_2, where the growth rates were 0.164 h^{-1} or 0.160 h^{-1} and OD reached 5.14 or 4.35, respectively, with complete VFA utilisation in 24 h (Figure 2B), the addition of H_2 resulted in dramatic growth inhibition and lower substrate utilisation reaching an OD of just 1.98 after 120 h cultivation (Figure 2C).

The release of CO_2 and gradual reduction of formate and other VFAs over time, coupled with the uptake of O_2 (Figure 2D) does indicate the presence of VFAs utilisation for growth, albeit slowly. Additionally, the gas utilisation profile showed release of CO_2 throughout the cultivation until 144 h where the cells began transitioning from heterotrophic to autotrophic growth (Figure 2D), suggesting little or no mixotrophy under these conditions.

3.4. Two-Step Mixotrophic Fermentation

As we have observed severe growth delay when 10 mM of each VFA was used in addition to CO_2 and H_2, we explored a two-step fermentation strategy, where *C. necator* H16 was initially grown with CO_2 and H_2 supplemented with only 5 mM of each VFA. We found that VFAs were all depleted after 24 h of cultivation, with the exception of hexanoic acid which was exhausted at 42 h (Figure 3A). Additionally, the growth rate and OD at 24 h were 0.126 h^{-1} and 3.7, corresponding to an 85.7% and 91.1% increase, respectively, compared to when 10 mM initial VFA was used. However, CO_2 uptake of up to 3.24 mmol/h (±0.403) can be observed at 36 h (Figure 3C), suggesting that cells have transitioned to autotrophic growth at this stage. After this initial growth phase, cells continued growing under autotrophic conditions (CO_2 and H_2) for an additional 6 h (Step 1, up to 48 h of cultivation).

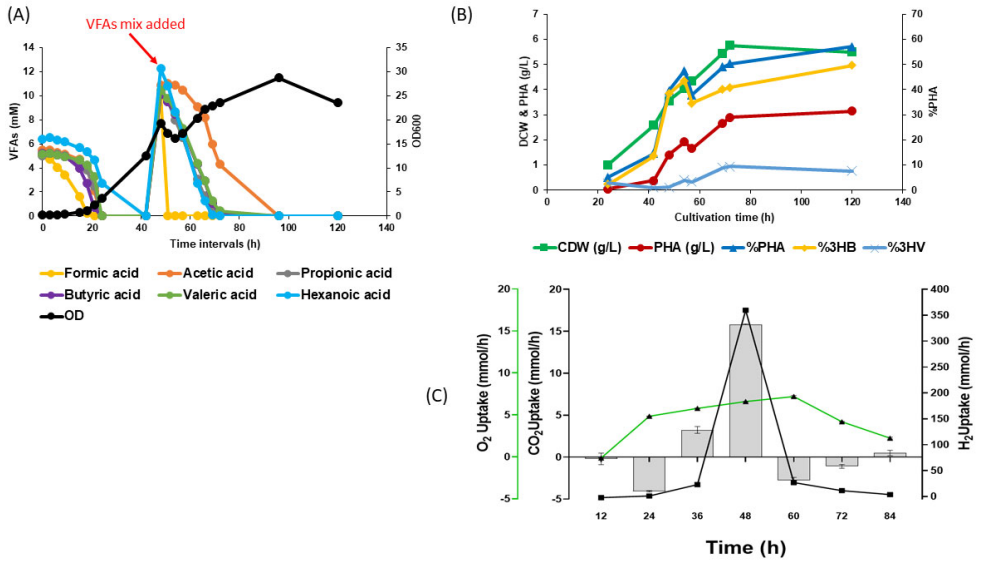


Figure 3. *C. necato*r H16 two-step mixotrophic fermentation in a bioreactor. Panel (**A**) growth (black line, OD600) and VFAs utilisation profile (colored lines corresponding to individual VFAs) under two step feeding regime.; Panel (**B**) cell dry weight (CDW) and PHA production profile under two step feeding regime. Biomass, wt% of PHA and PHA titer was monitored from 24 to 120 h of cultivation; Panel (**C**) gas utilisation profile under two step feeding regime. CO_2 and H_2 gas consumption were calculated as average of various time intervals. Legend: CO_2 uptake—grey bars, H_2 uptake—black line and O_2 uptake—green line.

During the first 48 h of cultivation, the biomass reached 3.6 g cdw/L and the accumulated PHA reached 1.4 g/L (38.9 wt% PHA/CDW) (Figure 3B). At this point, 10 mM of each VFA was added to the culture and cells were grown for a further 72 h (Figure 3A, up to 120 h of cultivation). We found that the "spiked" VFAs were all utilised within 48 h of cultivation. Surprisingly, the rate of VFAs utilisation was remarkably faster this time, suggesting little or no toxicity, compared to when 10 mM VFAs were added from the beginning of the cultivation (Figure 2A).

When 5 mM VFA was supplied at the beginning of the fermentation, the maximum CO_2 release was 4.07 mmol/h (±0.09) at 24 h, with only 0.96 mmol/h (±0.61) H_2 consumed. About 8 [(mol H_2)/(mol CO_2)] is required for CO_2 fixation [9], thus, the above result suggests little or no CO_2 utilisation within the first 24 h. However, when 10 mM VFA was supplied at 48 h, the maximum CO_2 produced was 2.76 mmol/h (±0.34) at 60 h, with hydrogen consumption of 26.95 mmol/h (±3.14). Additionally, at 120 h when the fermentation was stopped, the biomass concentration was 5.5 g cdw/L with a 57 wt% (g/gCDW) PHA content (Figure 3B). These are higher than the values recorded in the single VFA feeding regime.

3.5. Biomass and PHA Production from Different Batches of Anaerobic Digestate (AD) Derived VFAs

Having explored the options to produce PHA under mixotrophic conditions using synthetic VFAs, we tested the feasibility of using natural AD derived VFA mixtures under the same conditions. VFAs from 10 different batches of anaerobic digestates (ADs) were provided by ACEA Pinerolese Industrial Spa, Turin Italy, and used as a carbon source (at 3 g/L) in minimal media (MM described in material and methods) *C. necator* H16 fermentation experiments. To compensate for differences in batch volume, the amount of water in the media was reduced accordingly. For identification purposes, batches were labelled 1 to 10. The total VFA in the different batches varied from 15 to 40 g/kg and mostly comprised acetate, propionate, butyrate and hexanoate (Figure S1).

Significant batch-dependent differences in both biomass and PHA production were observed (Figure 4A, B), likely due to variation in VFAs composition (Supplementary Materials).

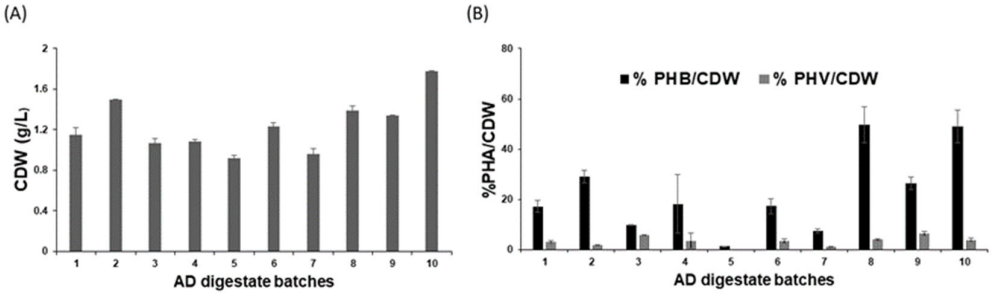


Figure 4. Biomass ((**A**) and PHA accumulation (**B**) of *C. necato*r H16 grown with Anaerobic Digestate (AD) derived VFAs. Cell dry weight (CDW (**A**), wt% PHB/CDW and wt% PHV/CDW (**B**) were measured in triplicates.

Biomass yields varied between 0.92 to 1.78 g cdw/L and 0.012 to 0.94 g/L whereas PHA production was from 1.31 to 53.59 wt%. Batch 2, 8, 9 and 10 resulted in 1.5, 1.39, 1.34 and 1.78 g/L cell dry weight and PHA productivity of 30.91, 53.59, 32.94 and 52.84 percentage cell dry weight, respectively. These results suggest that some of the AD derived VFA batches support a good level of biomass and PHA accumulation, whilst others resulted in significantly reduced levels.

3.6. PHA Production from AD Derived VFAs under Mixotrophic Fermentation

As a two-step mixotrophic fermentation was found to be the best feeding strategy for PHA accumulation when synthetic VFAs were used, this strategy was adopted for AD derived VFA fermentations. The four AD batches that supported higher biomass and PHA productivity (batches 2, 8, 9 and 10) were pooled to attain the highest possible PHA yields. The initial AD derived VFAs concentration in the medium was 2.5 g/L. After 48 h, an additional 5 g/L was added. Similar to results observed when using pure VFAs, lower CO_2 release with H_2 uptake was observed in the off-gas (Figure 5B), indicating mixotrophic growth. A maximum OD of 41 was reached after 120 h with a 44 wt% PHA content achieved (Figure 5A).

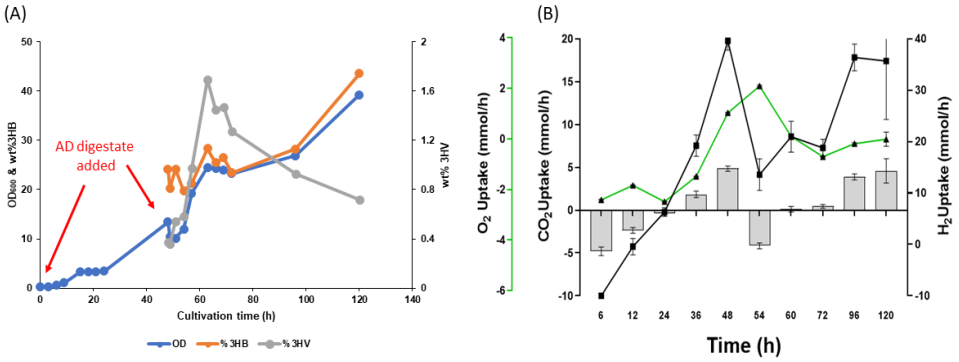


Figure 5. Two-step mixotrophic fermentation using AD derived VFAs, CO_2 and H_2. Panel (**A**) growth (blue line, OD600), wt% 3HB/CDW (orange line) and wt% 3HV/CDW (grey line) accumulation of *C. necator* H16 under two-step mixotrophic conditions in a bioreactor, using anaerobic digestate (AD) along with additional CO_2 and H_2 as carbon and energy source. Panel (**B**) gas consumption profile of cultures grown mixotrophically using AD derived VFAs, CO_2 and H_2. CO_2 and H_2 gas consumption were calculated as average of various time intervals. Legend: CO_2 uptake—grey bars, H_2 uptake—black line and O_2 uptake—green line.

The maximum 3HV content obtained was 1.69 wt% g/gCDW after 63 h accounting for 5.65% of total PHA. This dropped to 0.72 wt% g/gCDW, accounting for only 1.63% of total PHA at the end of the fermentation. The low proportion of 3HV in PHA was due to the low concentration of propionate and valerate present in the AD derived VFA mixture (Supplementary Materials).

4. Discussion

Circular economy strategies are essential to driving decarbonisation, and as such, utilisation of CO_2 and other waste carbon sources as feedstocks to replace petrochemical based chemical synthesis are at the heart of these efforts. Utilisation of the double waste stream, CO_2 and VFAs generated during AD would allow carbon loop closure and circularity. Several drawbacks still hinder the commercial uptake of aerobic autotrophic fermentations, such as the use of explosive H_2 and O_2 mixtures and mass-transfer limitations, especially of O_2. Additionally, high H_2 requirements for aerobic CO_2 fixation also limit the commercialisation of this technology due to the current high cost of electrolytic H_2. Mixotrophy, particularly with VFAs could potentially reduce the need for electrolytic H_2 and simultaneously lead to the incorporation of other HA monomers such as HV into PHA structures. Additionally, utilisation of waste streams as sources of carbon and energy should reduce the cost of PHA production. During anaerobic digestion, both CO_2 and VFAs are produced in large quantities, and these can be diverted towards PHA production in an integrated system. Here, we have shown for the first time, the possibility of mixotrophic growth of *C. necator* H16 using AD derived VFAs as added carbon and energy source.

VFAs have been previously shown to be toxic to a wide range of microorganisms including *Escherichia coli*, *Photobacterium phosphoreum* and *Pseudomonas aeruginosa* at various concentrations and physicochemical conditions [21–24]. However, no data exist in the literature for the toxicity profile of all six VFA used in this study. Therefore, a toxicity assay was carried out and results showed that although *C. necator* was able to use all six VFAs tested, their toxicity increased with concentration and their chain length. These results are consistent with those observed in engineered *Ralstonia eutropha* Re2133, *E. coli*, *Photobacterium phosphoreum* and *Pseudomonas aeruginosa* [21–24,29]. No growth inhibition was observed for formic acid for concentrations up to 50 mM, rather the cells reached the stationary phase earlier at much lower OD. The previous report has also shown lower biomass when *C. necator* is grown in formate compared to other carbon sources including butyrate and fructose [30]. This was attributed to carbon loss in the form of CO_2, with low carbon conversion to biomass at 0.16 Cmole Cmole-1, compared to either fructose at 0.63 or butyrate at 0.64 Cmole Cmole-1. Undissociated VFA can penetrate cell membrane easily and dissociate within cells' cytoplasm inhibiting growth [31] by disrupting the proton gradient across the cell and blocking ATP synthesis [32]. On the other hand, at higher pH, VFAs can occur in their dissociated form [33,34] leading to an increase in ionic strength at higher concentrations and eventually resulting in cell lysis [23,32]. As minimal or nonsignificant cell toxicity or growth inhibition was observed at 10 mM of each VFA, we opted to use this concentration for each VFA in PHA production in bioreactors from synthetic VFA mix.

Prior to bioreactor studies, we assessed PHA production from individual VFAs using 50 mM carbon equivalent. We observed that while cultures grown with butyric acid as the sole carbon source, had the highest biomass yield, cultures grown with valeric acid had the highest PHA yield (g/L) and content (wt% g/gCDW). PHA in the latter was composed mostly of 3HV and very little 3HB (Table 1), in line with previous reports [32,35].

Next, we carried out the mixotrophic feeding experiments, using minimal medium supplemented with a mixture of C1–C6 VFAs (10 mM each) under three different conditions, and we observed significant variation in growth and PHA profiles under these conditions. A higher OD and PHA accumulation was observed in the presence of CO_2 as an additional carbon source, compared to VFA only cultures. These increases suggest CO_2 uptake as an additional source of carbon with formate providing electrons for CO_2 fixation via the Calvin cycle [36]. Under both conditions, the maximum PHA content observed was 10.88 wt% g/gCDW, significantly lower than the over 80% PHA [37] this bacterium is known to accumulate. Additionally, upon complete utilisation of VFAs, a continuous decline in PHA composition was observed in both conditions, hence the fermentation was stopped at 24 h. The PHA yield was expected due to the limited carbon supply, resulting in a low C:N ratio of 11.5 which has been previously reported to favour biomass and not PHA accumulation [38]. Indeed, Yang et al. studied PHA production from organic acids and reported little or no PHA produced at C:N ratios below 20 and optimal PHA yield per CDW at C:N ratio of 80 [39]. The low C:N ratio can also explain the decline in PHA content upon complete VFAs utilisation, as carbon limitation is known to trigger PHA re-utilisation in *C. necator* [26,40].

In another experiment, H_2 was supplied in addition to CO_2 and VFA to provide an additional source of energy. Interestingly, we observed that the addition of H_2 resulted in dramatic growth inhibition and lower substrate utilisation. Previous research has shown that species of *C. necator* can reach high cell densities when grown autotrophically in the presence of CO_2 and H_2 [18]. Additionally, in a separate study (results not shown), we have attained OD greater than 80 in batch cultures after 96 h post-inoculation. Substrate toxicity is known to result in lower growth rates and OD values [24]. Thus, with approximately 87% reduction in growth rate and up to 93% reduction in OD when compared to reactors without H_2, we believe that the addition of H_2 either increases the toxicity of VFA or limits the VFA uptake under these conditions. We postulate that the inability of the cells to effectively utilize H_2 and CO_2 for growth autotrophically under these conditions

is due to the repression of the hydrogenases by VFAs. This is because, research has shown that organic substrate can limit hydrogenase expression to different degrees, depending on the substrate [41]. However, it remains unclear why the cells could not successfully utilise the VFAs until 166 h of cultivation. One possible explanation is that H_2 exerts a possible regulatory inhibition of VFA uptake/transport and/or CoA activation, by an unknown mechanism. This is because formate, the only VFA utilised within 96 h, does not need to be activated and is readily assimilated. While other VFAs must be activated into their corresponding acyl-CoA forms by the action of fatty acid-CoA synthetases (or fatty acid-CoA ligases) in an ATP dependent manner. In *C. necator*, it has been shown [42] that the presence of acetate and levulinic acid induced the level of acetate kinase (AckA) and phosphotransacetylase (Pta), as well as acetyl-CoA synthetase (Acs), enzymes involved in acetate metabolism when this is present at low (Acs pathway) or high concentrations (Pta-AckA pathway) in the growth medium. However, how the presence of molecular H_2 in addition to acetate (or other VFAs) is affecting the expression of these enzymes is again unknown. These novel findings will require further investigation using a systems level approach to elucidate the effect of H_2 on organic acid accumulation or vice versa.

To mitigate the combined effect of CO_2, H_2 and VFA on cell growth, PHA accumulation and substrate utilisation, a two-step fermentation strategy was performed. In the first step, 5 mM of VFA mixture was used, upon complete VFA utilisation, a further 10 mM of VFA mixture was added in the second step. CO_2 and H_2 gases were continuously sparged into the reactor in both steps. Effective utilisation of H_2 and CO_2 was observed under the two-step feeding regime. In the second stage, an increase in hydrogen consumption coupled with reduced CO_2 levels in the off-gas was observed, suggestive of mixotrophic growth, where cells were utilising both CO_2 alongside the VFA. Effective utilisation of H_2 and CO_2 in this two-step feeding regime compared with the single feeding regime suggests reduced repression of hydrogenases at lower concentrations of VFAs. It has been previously noted that the transcription and translation of hydrogenase genes occurs within 5 h post induction, after which active enzymes are produced from a pool of inactive precursors [43]. This may explain why H_2/CO_2 utilisation continued even when 10 mM of VFA was added in the second feeding regime. Furthermore, a remarkable increase in VFA utilisation rates was observed in the two-step feeding regime compared to the single feeding regime even at 10 mM VFA concentration. Kedia et al. (2014) had similar findings, with increased VFA uptake and PHA production observed in *C. necator* in a continuous feeding regime compared to a one time feeding [44]. This could be due to an increase in VFA tolerance, following initial adaptation and subsequent increase in cell population in exponential growth phase leading to effective distribution and utilisation of available VFAs. In addition to the remarkable increase in VFA utilisation, increased H_2 uptake and lower CO_2 release was also observed in the second stage of the fermentation compared to the first stage. The increase in hydrogen consumption coupled with reduced CO_2 levels in the off-gas indicates that the cells are growing mixotrophically, utilising available CO_2 alongside the VFA

Following process optimisation using synthetic VFAs, mixotrophic growth of *C. necator* in AD derived VFAs was also assessed. Firstly, the ability of *C. necator* to utilize the VFAs present in the various batches of AD was studied. It was found that at similar VFA concentrations, cell growth and PHA accumulation varied considerably in the different AD batches. This was attributed to the variation of VFA composition in the different batches as previous reports have shown that VFA composition affects both biomass, PHA yields and PHA composition. Higher PHA content is observed in the presence of butyrate and acetate compared with acetate and propionate [45], with increasing HV content observed as valerate or propionate concentrations increase [32,45]. Correlation analysis to investigate the effect of VFA composition in the various AD batches on overall PHA production or HV content was carried out. A positive correlation coefficient of 0.77 was observed between valeric acid composition in AD, and HV content in PHA, with a p-value < 0.05 (Figure S2), indicating that HV content is dependent on valeric acid composition.

However, no correlation was found between propionic acid composition and HV content. This is likely because the amount of propionic acid in the AD batches was low. Additionally, it has been shown that the majority of propionyl-CoA is converted to pyruvate and then decarboxylated to acetyl-CoA and CO_2 with only 6% condensed with acetyl-CoA to eventually give HV [46]. Thus, the low propionic acid composition coupled with its low conversion to HV may account for the lack of correlation between HV content and propionic acid composition in the AD batches. Finally, the optimised two-step mixotrophic fermentation was employed with AD derived VFAs instead of synthetic VFAs. A higher OD with a much lower PHA content was observed under this condition compared with growth on synthetic VFA. The increase in OD and subsequent reduction in PHA content can be attributed to the presence of additional nitrogen source in the AD. Since the nitrogen content of different AD batches is variable, future fermentation experiments should, therefore, be focused on optimising cultivation conditions for higher PHA yield. This can be achieved by exploring phosphate and/or oxygen limitation. It can also be achieved by reducing or eliminating the nitrogen in the minimal media.

In conclusion, the utilisation of AD-derived VFAs and CO_2 for the mixotrophic production of PHAs presents an exciting opportunity to produce copolymers with desirable physicochemical properties. The fact that AD systems can be fine-tuned to yield desired compositions [47], also presents an exciting opportunity to produce PHA with varying proportions of HV, thus further improving the quality of the synthetised PHA and potentially tailoring PHA obtained from mixotrophic CO_2 and AD derived VFA feeding for specific applications. Here, a mixotrophic growth strategy has been established using AD derived VFAs and CO_2; however, future fine-tuning of process parameters will be necessary to optimise PHA productivity and HV content under these conditions. These can be directed towards continuous monitoring of C:N ratios, adapting and optimising conditions for the growth phase and PHA production phase when using the mixture of VFAs and CO_2.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/fermentation8030125/s1, Figure S1: Composition of VFAs from 10 different batches of AD digestate provided by ACEA; Figure S2: Pearson correlation analysis showing the relationship between PHV content (wt%) and valerate concentration (g/l) from different batches of anaerobic digest provided by ACEA. Only 8 batches were considered for this analysis due to the lack of valeric acid in two of the batches.

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