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Advanced PHB fermentation strategies with CO_2-derived organic acids

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ABSTRACT

Over the past decade, formic acid and acetic acid have gained increasing attention as alternative feedstocks for poly-3-hydroxybutyrate (PHB) production as these potentially CO_2-derived molecules are naturally assimilated by *Cupriavidus necator*. Both organic acids were individually evaluated in fed-batch fermentations at bioreactor scale. Acetic acid was revealed as the most promising carbon source yielding 42.3 g L^{-1} PHB, whereas no significant amount of PHB was produced from formic acid. Hence, acetic acid was further used as the substrate during process intensification. Key performance characteristics, including process stability, PHB titer, and productivity were optimized by introducing NH_4-acetate as the nitrogen source, extending the growth phase, and implementing a repeated fed-batch procedure, respectively. These advanced fermentation strategies resulted in the establishment of a stable fermentation process reaching 58.5 g L^{-1} PHB, while doubling the productivity to 0.93 g L^{-1} h^{-1} PHB.

1. Introduction

While plastics form a cornerstone in our contemporary society, the ever-increasing use of these mostly fossil-based materials has been leading to the emission of 1.7 $GtCO_2eq/y$, corresponding to roughly 3.8% of the global greenhouse gas (GHG) emissions (Zheng and Suh, 2019). In addition, their disposal exerts a disastrous impact on our environment as a staggering 8 million tons of plastic waste ends up in the world's oceans every year with the associated detrimental effect of toxic microplastics on aquatic life and human health (Campanale et al., 2020).

In this context, biodegradable plastics derived from sustainable resources have attracted increasing attention as appropriate substitutes. Poly-3-hydroxybutyrate (PHB) is a microbially produced bioplastic and shows excellent biodegradability under various conditions (Meereboer et al., 2020). Its applications are diverse, ranging from biodegradable packaging to medical components such as prostheses, owing to their biocompatibility, or drug delivery vehicles, as they do not generate toxic substances upon degradation (Althuri et al., 2013; Koller et al., 2010). Although industrial PHB production has been achieved by several companies including Biomer (Germany), BASF (Germany) and Kaneka (Japan), large-scale commercialization is currently still hampered. This is mainly due to the high overall production cost, of which up to 50% has been ascribed to the requirement of high purity substrates (Koller et al., 2017; Sun et al., 2007).

Therefore, industrial side-streams including lignocellulosic waste, waste glycerol and wastewater have been investigated for PHB production (Annamalai et al., 2017; Cavalheiro et al., 2009; Khardenavis et al., 2007). Although these feedstocks imply more extensive upstream and downstream processing and are typically characterized by a lower conversion yield, promising results have been obtained (Du et al., 2012; Sirohi et al., 2020). Alternatively, already explored several decades ago and currently gaining renewed attention as a carbon capture and utilization (CCU) technology, CO_2 can serve as a carbon source for PHB production, thereby closing the bioplastic carbon cycle (Garcia-Gonzalez et al., 2015; Schlegel and Eberhardt, 1972).

Cupriavidus necator (formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus,* and *Wautersia eutropha*) is a Gram-negative strict aerobic bacterium and has been extensively studied for PHB synthesis, both heterotrophically and autotrophically (Lee, 1996; Nangle et al., 2020). However, the gaseous feedstock, consisting of CO_2, H_2, and O_2, required for autotrophic cultivation conveys two important limitations: (i) the gas transfer into the culture is limited, and (ii) the O_2 concentration should

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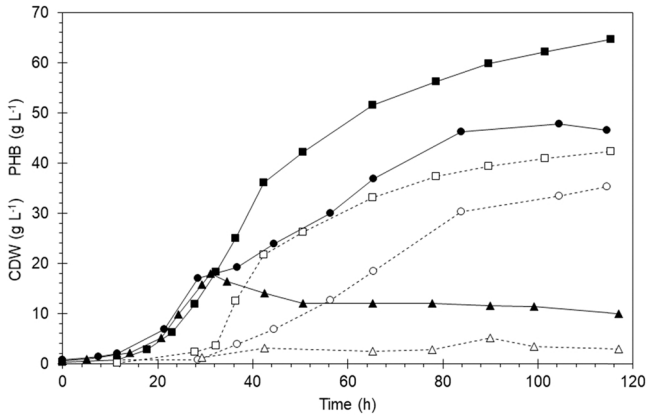

Fig. 1. CDW (full line) and PHB (dotted line) concentration of fed-batch fermentations with a growth phase on glycerol and a PHB accumulation phase on glycerol (●,○), formate (▲,△), or acetate (■,□).

Table 1
Comparison of feedstocks for PHB production in terms of PHB yield and CO_2 fixation based on stoichiometric equations.

Substrate (S)	Stoichiometric reaction to PHB	PHB yield (gPHB/gS)	CO_2 fixation (gCO_2/gS)
CO_2/H_2	$4\ CO_2 + 33\ H_2 + 12\ O_2 \rightarrow C_4H_6O_2 + 30\ H_2O$	0.36	0.73
Glycerol	$2\ C_3H_7O_3 + 2.5\ O_2 \rightarrow C_4H_6O_2 + 5\ H_2O + 2\ CO_2$	0.47	-0.48
Formic acid	$33\ CH_2O_2 + 12\ O_2 \rightarrow C_4H_6O_2 + 30\ H_2O + 29\ CO_2$	0.06	0.12[a]
Acetic acid	$3\ C_2H_4O_2 + 1.5\ O_2 \rightarrow C_4H_6O_2 + 9\ H_2O + 2\ CO_2$	0.48	0.98[a]

[a] Given that formic acid and acetic acid can be produced from CO_2 via electrochemical ($CO_2 + H_2 \rightarrow CH_2O_2$) and biological ($2\ CO_2 + 4\ H_2 \rightarrow C_2H_4O_2 + 2\ H_2O$) conversion of CO_2, respectively.

be kept below the lower level of explosion (Teixeira et al., 2018). To circumvent these issues, CO_2-derived liquid feedstocks, such as formic acid and acetic acid, have recently been introduced as best-of-both-worlds alternatives (Kiefer et al., 2020; Satanowski and Bar-Even, 2020). Indeed, formic acid and acetic acid can be obtained from CO_2 via electrochemical or thermo-catalytical reduction and microbial conversion by acetogenic bacteria, respectively (Kantzow et al., 2015; Rumayor et al., 2018). In addition, their assimilation by *C. necator* has been demonstrated.

Since it is known that short-chain organic acids are toxic to *C. necator* at elevated concentrations, a fed-batch fermentation is required to produce PHB from these feedstocks (Vázquez et al., 2011). This is usually achieved by a pH-static feeding strategy in which the acidic substrate is used to control the pH of the bioreactor. Grunwald et al. (2015) demonstrated this for formic acid and achieved a final biomass concentration of 5.4 g L^{-1} with a PHB-negative *C. necator* strain. Using acetic acid, Garcia-Gonzalez and De Wever (2018) reported the production of 43 g L^{-1} PHB by *C. necator* with a productivity of 0.37 g L^{-1} h^{-1} PHB. These are, to the best of our knowledge, the highest obtained results to date for the cultivation of *C. necator* purely on formic acid or acetic acid.

Conclusively, while a proof-of-concept for the assimilation of both formic acid and acetic acid by *C. necator* has been delivered, the true potential of these CO_2-derived organic acids to produce PHB remains to be revealed. The objectives of this work, therefore, are to investigate both acids as alternative feedstocks for *C. necator*, aiming to develop an effective, readily scalable, and industrially feasible strategy for PHB production.

2. Materials and methods

2.1. Bacterial strain

Cupriavidus necator DSM 545 was used for all cultivations and was acquired from the Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (Germany). Stock cultures of 1.5 mL late exponential culture in Lennox broth (LB) with 20% glycerol were prepared and stored at −80 °C in cryovials.

2.2. Media and inoculum preparation

LB was used as the seed medium for the first preculture. For subsequent precultures, a minimal medium was used consisting of the carbon source (glycerol or Na-acetate) and the following compounds (per liter distilled water): 3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 4.47 g $Na_2HPO_4{\cdot}2H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, and 1 mL trace elements solution. The trace elements solution consisted of (per liter distilled water): 10 g $FeSO_4{\cdot}7H_2O$, 2.25 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}5H_2O$, 2 g $CaC1_2{\cdot}2H_2O$, 0.23 g $Na_2B_4O_7{\cdot}10H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}$, and 10 mL 37% HCl. The fermentation medium had the following composition (per liter distilled water): 17 g glycerol or 1.39 g Na-acetate, 4 g $(NH_4)_2SO_4$, 13.3 g KH_2PO_4, 1.2 g $MgSO_4{\cdot}7H_2O$, 1.87 g citric acid, and 10 mL trace elements solution.

Stock cultures were used to inoculate the first preculture by transferring 1.5 mL from a cryovial to 50 mL of LB medium in 250 mL flasks, which were incubated in an orbital shaker at 200 rpm (orbit 19 mm) and 30 °C for 24 h. The next precultures were cultivated on the carbon source used during the growth phase of the subsequent fed-batch fermentation. When using glycerol, successive sub-culturing was performed five times in minimal medium supplied with 17 g L^{-1} glycerol to ensure adaptation of the microorganisms to the glycerol substrate (4% v/v inoculum) (Mozumder et al., 2014a). The last subculture was used to inoculate the final preculture step, which was performed in 500 mL baffled flasks containing 100 mL of medium for 24 h (3% v/v inoculum). When using acetic acid, the first preculture was used to inoculate a flask of 250 mL containing 50 mL of minimal medium supplied with 1.39 g L^{-1} of Na-acetate (3% v/v inoculum). After 8 h of cultivation, it was transferred to 500 mL baffled flasks containing 100 mL of medium supplied with 4.17 g L^{-1} Na-acetate (3% v/v inoculum). In both cases, 375 mL of the final preculture was used to inoculate the fermenter (12.5% v/v inoculum).

2.3. Fed-batch fermentations

Fed-batch fermentations were performed in 7.5 L autoclavable glass bench-top bioreactors (Labfors 4, Infors HT, Switzerland) with an initial working volume of 3 L. All runs were carried out as single experiments with continuous monitoring and control of key process prarameters for close follow-up, hence eliminating important sources of variability. The feed solutions consisted of 500 g L^{-1} glycerol, 500 g L^{-1} formic acid or 500 g L^{-1} acetic acid, unless noted otherwise. Temperature was kept at 30 °C. pH was controlled at 6.8 via the addition of 1 M H_2SO_4 and 1 M NaOH, except for when pH-static feeding was applied. The dissolved oxygen (DO) was controlled at 30% by adjusting the stirrer speed (200–1200 rpm) and the airflow (0.06–2 vvm). All online bioreactor parameters were controlled by Eve Bioprocess Software (Infors HT, Switzerland). The O_2 and CO_2 concentrations in the off-gas were measured with a Tandem PRO (Magellan Biotech, UK) gas analyzer and used to calculate the oxygen uptake rate (OUR). Samples were collected at regular time intervals and analyzed according to Section 2.4.

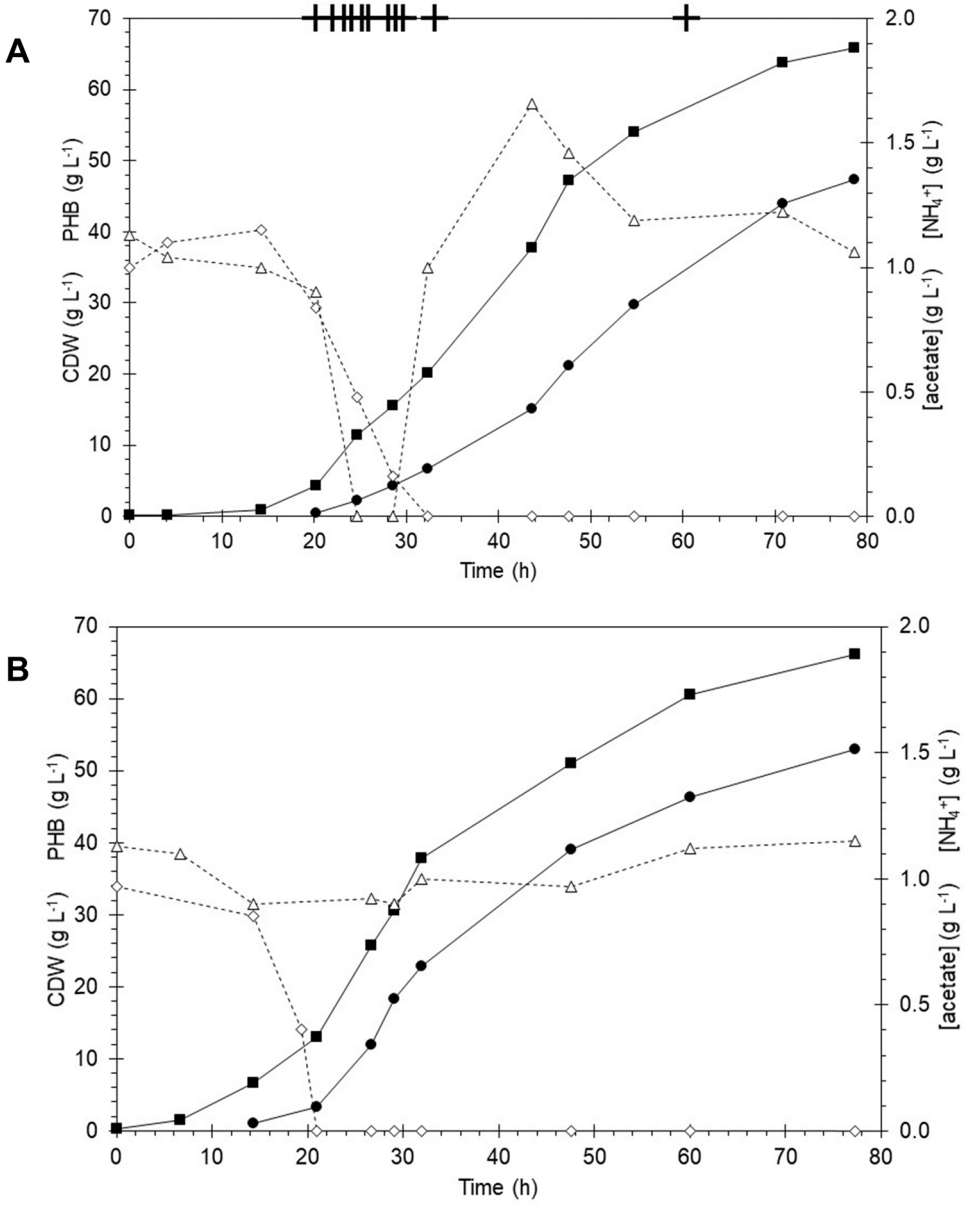

Fig. 2. CDW (■), PHB (●), acetate (△), and NH_4^+ (◇) concentration of two fed-batch fermentations on acetic acid through pH-static feeding with a different nitrogen source in the feed: $(NH_4)_2SO_4$ (A) and NH_4-acetate (B). The points at which acetate was depleted and Na-acetate was added to reinitiate the pH-static feeding are indicated (✚).

Table 2
Strategy for the inclusion of a linear growth phase before PHB accumulation.

Phase	Feeding strategy	C/N ratio	Terminated by
1) Exponential growth	pH-stat	10	Aeration at maximum (OUR=OTR)
2) Linear growth	DO-stat	10 to 90[a]	Nitrogen depletion
3) PHB accumulation	pH-stat	90	

[a] Switch to 90 near the end of this phase to decrease nitrogen concentration.

2.4. Analytical methods

The biomass concentration, or cell dry weight (CDW) (g L^{-1}), was determined gravimetrically. To that end, 5 mL of the culture broth was centrifuged at 4700 g for 10 min. The resulting cell pellet was washed with physiological water, resuspended in distilled water and transferred to a moisture analyzer (MA37, Sartorius, Germany), which allowed to dry the sample to a constant weight. The PHB content of the cells was determined through thermogravimetric analysis (TGA) and is expressed as g PHB per g CDW (%). Therefore, a cell pellet was dried for at least 24 h at 60 °C and transferred to tarred platinum pans on the thermogravimetric analyzer (TGA 550, TA instruments, USA). It was heated from 25° to 500 °C at a rate of 5 °C min^{-1} under a nitrogen flow of 50 mL min^{-1}. The weight loss within the temperature range of 250–290 °C was assigned to PHB. For quantification, a calibration was performed with a more extensive method, consisting of the hydrolysis of the dried samples with 2 M NaOH at 90 °C and analysis of the resulting 3-hydroxybutyric acid monomer via HPLC. Concentrations of formate, acetate, and glycerol were determined by HPLC analysis (1260 Infinity II LC System, Agilent, USA) with a Metacarb 67H column (300 × 6.5 mm) at 40 °C, a mobile phase of 2.5 mM H_2SO_4 at 0.8 mL min^{-1} and a refractive index detector (RID). The concentration of ammonium (NH_4^+) was measured

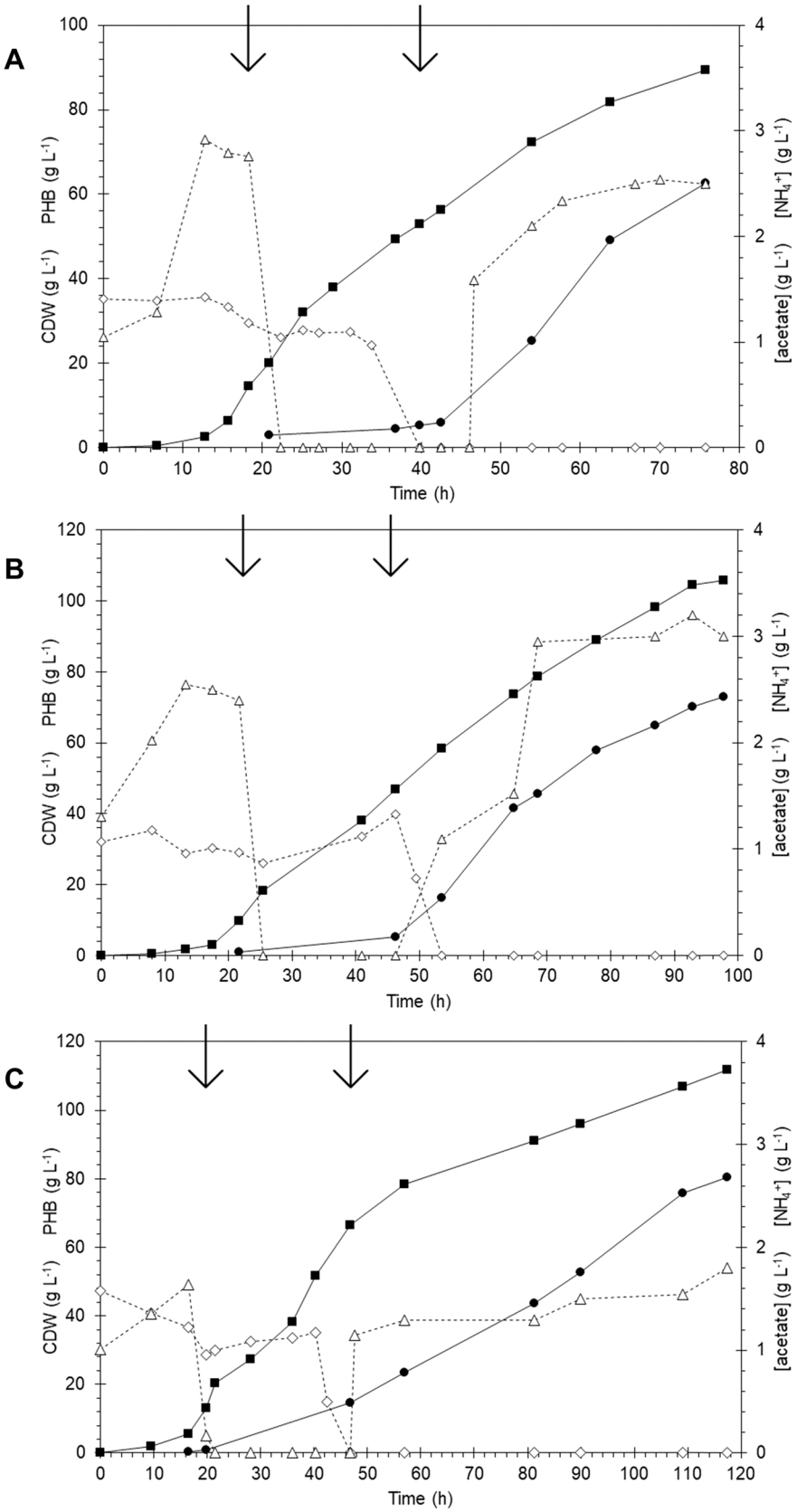

Fig. 3. CDW (■), PHB (●), acetate (△) and NH_4^+ (◇) concentration of fed-batch fermentations on acetic acid. The first arrow depicts the switch from exponential (phase 1) to linear growth (phase 2) by stopping pH-static feeding and starting feeding at a constant rate. The second arrow depicts the initiation of PHB accumulation (phase 3) by nitrogen depletion.

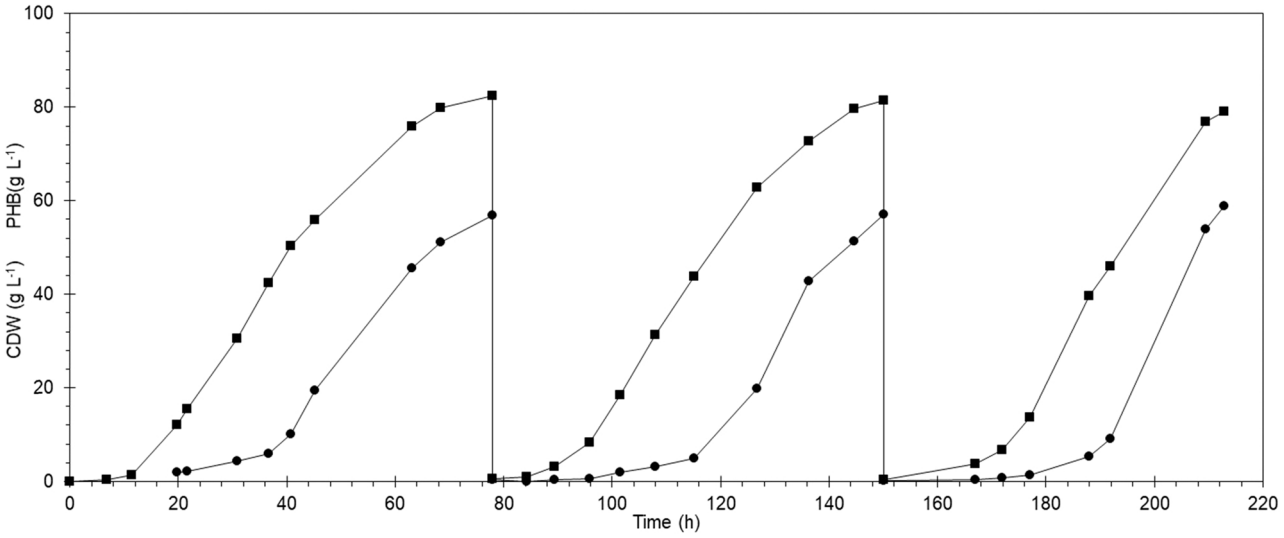

Fig. 4. CDW (■) and PHB (●) concentration for three cycles of a repeated fed-batch fermentation of *C. necator* on acetic acid.

with an ion-selective electrode (2900D Biochemistry Analyzer, YSI, USA).

3. Results and discussion

3.1. CO_2-derived organic acids as substrates for PHB production

Although both formic acid and acetic acid have been described as promising CO_2-derived substrates for *C. necator* (Garcia-Gonzalez and De Wever, 2018; Grunwald et al., 2015), a distinct comparison has not been made to date. Therefore, we evaluated the performance of both organic acids and compared this to glycerol as a benchmark by performing fed-batch fermentations. These consisted of an equivalent growth phase on glycerol and a subsequent PHB accumulation phase on formic acid, acetic acid or glycerol, which was induced by nitrogen limitation (Fig. 1).

During the growth phase, sufficient nitrogen was supplied by using NH_4OH for pH control, keeping the NH_4^+ concentration between 0.5 and 1.5 g L^{-1}. After around 24 h of exponential growth, when the biomass concentration exceeded 10 g L^{-1} CDW, the base was switched to NaOH, causing the depletion of nitrogen at around 18 g L^{-1} CDW. At this point, the OUR reached its maximal value of 120 mmol L^{-1} h^{-1}. To assess the conversion of formic acid and acetic acid into PHB, glycerol addition was stopped, and 1 g L^{-1} of formate or acetate was added as their sodium salt. Both carboxylates were consumed immediately upon their addition, which was observed by a rise in pH. This initiated pH-static feeding of the corresponding organic acids, while the formate and acetate concentration remained around 1 g L^{-1}. Both the substrate and ammonium concentration were measured at regular timepoints assuring sufficient carbon supply and nitrogen limitation to stimulate PHB accumulation (data not shown).

Although formate and acetate were consumed at a similar average rate of 1.2 g L^{-1} h^{-1}, a large difference was observed with respect to PHB production. When using formic acid, no significant amount of PHB was accumulated, while the biomass concentration decreased from 18.0 to 10.0 g L^{-1} CDW. To confirm this low performance, the experiment was repeated, and in addition, the effect of switching to the PHB accumulation phase at lower biomass concentrations, namely at 15.7 and 11.9 g L^{-1} CDW, was assessed (data not shown). Nevertheless, although formate supply and nitrogen limitation were assured during the second phase of all experiments, also no significantly higher amounts of PHB were synthesized in these cases. In contrast, when using acetic acid, the biomass and PHB concentration increased from 18.2 to 64.6 g L^{-1} CDW and from 3.6 to 42.3 g L^{-1} PHB. Notably, these results appear to exceed the benchmark fermentation on glycerol, in which the biomass and PHB concentration increased from 19.2 to 46.6 g L^{-1} CDW and from 3.8 to 35.3 g L^{-1} PHB.

The fact that acetic acid is used more efficiently than formic acid could be anticipated by considering the different assimilation routes. The stoichiometric equations and theoretical PHB yields are presented in Table 1. Formate is indirectly assimilated, as it is first oxidized by formate dehydrogenase to deliver reducing equivalents, after which the resulting CO_2 is used in the Calvin cycle. This cycle is naturally the most dominant pathway for aerobic CO_2-fixation and is known to be energetically inefficient, demanding eight reducing equivalents to incorporate one CO_2 (Bar-Even et al., 2012). In autotrophic fermentations, this high energy requirement is met by the supply of additional H_2 gas, thereby providing a substrate with a high energy-to-carbon ratio (Garcia-Gonzalez et al., 2015). However, in the case of formic acid, this ratio is fixed and not in line with the energy-demanding Calvin cycle, which could explain the lack of PHB accumulation. More specifically, the oxidation of 33 formic acid molecules is required to fix four CO_2 molecules into one PHB monomer, while 29 CO_2 molecules are emitted (0.84 g CO_2 per g formic acid). In contrast, acetate is directly converted by acetyl-CoA synthetase into acetyl-CoA, the precursor for PHB (Yamane, 1993). This route is thus more efficient requiring only three acetate molecules to provide one PHB monomer, accompanied by the emission of two CO_2 molecules (0.48 g CO_2 per g acetic acid). Off-gas analysis confirmed these routes experimentally, measuring ratios for carbon emitted as CO_2 of 83% and 50% in the case of formic acid and acetic acid, respectively. Besides the difference in assimilation routes, the lack of PHB production on formic acid could also be caused by a different regulation of the metabolism when switching from growth to PHB synthesis, which could be elucidated by future transcriptomics or proteomics studies (Jahn et al., 2021).

Since the less efficient assimilation of formic acid leads to more CO_2 emission, the carbon fixation potential of the complete process will be lower, thereby hampering its exploitation as CCU method. In this respect, novel synthetic routes enabling more efficient formate incorporation, such as the reductive glycine pathway, offer promising alternatives (Claassens et al., 2020). On the other hand, the more efficient acetic acid assimilation results in a higher carbon fixation potential. Yet, in order to make a full comparison, also the CO_2-based production of both substrates should be taken into account. Claassens et al. (2019) estimated the energy efficiency of electrochemical formate production at 50%, whereas for acetate production, consisting of acetogenic

conversion at 75% using H_2 generated at 70%, an efficiency of 53% was calculated. When combined with the subsequent conversion efficiency by *C. necator* of 40% for formic acid and 50% for acetic acid, the overall energy efficiency was estimated at 20% and 26%, respectively (Claassens et al., 2019). Therefore, although it requires the implementation of an additional fermentation step, acetic acid indeed seems to have great potential as CO_2-derived feedstock for PHB production by *C. necator*.

3.2. Acetic acid as a promising alternative feedstock for PHB production

Since acetate was found to be a propitious CO_2-derived carbon source to produce PHB with *C. necator*, it was studied in more detail. Different strategies were elaborated aiming to develop an intensified process in which acetic acid was used as the substrate both for growth and PHB accumulation.

3.2.1. Increasing process stability with NH_4-acetate as nitrogen source

In order to supply sufficient nitrogen for growth during pH-static feeding of acetic acid, a nitrogen salt is typically added to the feed solution in a particular C/N ratio. The importance of this ratio has been extensively investigated suggesting 10 (330 g L^{-1} acetic acid and 73 g L^{-1} $(NH_4)_2SO_4$) and 90 (660 g L^{-1} acetic acid and 16 g L^{-1} $(NH_4)_2SO_4$) as optimal ratios for growth and PHB accumulation, respectively (Garcia-Gonzalez and De Wever, 2018). However, in line with the report of Huschner et al. (2015), this strategy was found to suffer from instability. Indeed, when using those feed solutions, the acetate concentration decreased throughout the fermentation, requiring frequent additions of Na-acetate to reinitiate the pH-static feeding mechanism (Fig. 2A).

Conveniently, when NH_4-acetate was examined as an alternative nitrogen source, using the same C/N ratios of 10 for growth (660 g L^{-1} acetic acid and 212 g L^{-1} NH_4-acetate) and 90 for PHB accumulation (660 g L^{-1} acetic acid and 19.3 g L^{-1} NH_4-acetate), the acetate concentration remained stable around 1 g L^{-1} during three days of fermentation (Fig. 2B). Hence, besides maintaining low acetate concentrations, this strategy also enabled a continuous supply of carbon, without the need for Na-acetate additions. Consequently, to increase process stability, NH_4-acetate was used as the nitrogen source for all the following fermentations. In addition, this strategy also increased the productivity from 0.60 to 0.69 g L^{-1} h^{-1} PHB.

3.2.2. Improving PHB production by linear extension of the growth phase

Since the amount of accumulated PHB is directly proportional to the number of cells, a high cell density is desired to further improve PHB production (Ienczak et al., 2011). However, when a pH-static feeding strategy is applied, growth will be oxygen-limited. More specifically, the unrestricted supply of substrate during pH-static feeding results in exponential growth and an increasing OUR. This inevitably comes to an end when the OUR reaches the maximum oxygen transfer rate (OTR) of the bioreactor. The resulting oxygen limitation will restrict growth and cause the undesired, early induction of the PHB accumulation phase, as described by Garcia-Gonzalez et al. (2015).

Hence, in this case, the OTR of the fermentation equipment determines the maximum biomass concentration that can be reached by exponential growth (CDW_{exp}) before PHB accumulation initiates. In fact, CDW_{exp} (g) can be calculated from the OTR (mmol L^{-1} h^{-1}), the oxygen yield coefficient ($Y_{CDW/O2}$) (g mol^{-1}) and the maximum specific growth rate (μ_{max}) (h^{-1}), as shown in Eq. 1. When using acetic acid as the substrate, $Y_{CDW/O2}$ equals 27 g mol^{-1} (Garcia-Gonzalez and De Wever, 2018) and μ_{max} was derived from the pH-static growth phase as 0.25 h^{-1}.

$$CDW_{exp} = \frac{OTR \times Y_{CDW/O_2}}{\mu_{max}} \quad (1)$$

Indeed, at the end of the pH-static growth phase on acetic acid, when aeration was at its maximum and an OTR of 120 mmol L^{-1} h^{-1} was observed, the biomass concentration reached 13.0 g L^{-1} CDW, which is in line with Eq. 1. At that point, nitrogen limitation was imposed to induce PHB accumulation and avoid oxygen limitation. This resulted in a final biomass and PHB concentration of 66.2 g L^{-1} CDW and 53.0 g L^{-1} PHB over 77.3 h (Fig. 2B).

Yet, aiming to increase PHB production, the premature shift to PHB accumulation caused by oxygen limitation at 13 g L^{-1} CDW should be prevented. A higher biomass concentration may be reached by prolonging the exponential growth phase, which in turn can be achieved through a higher OTR. However, the latter requires advanced techniques such as improved reactor design, operation at elevated pressure or supply of pure oxygen, which all come with a substantial cost or are industrially less feasible (Garcia-Ochoa and Gomez, 2009). Therefore, an alternative strategy to support growth to higher biomass concentrations was developed (Table 2). The novel strategy encompasses the extension of the pH-static exponential growth phase (phase 1) with a DO-static linear growth phase (phase 2). The latter involves restricted addition of the substrate at a constant feed rate, to avoid oxygen limitation and thus also the premature shift to PHB accumulation. This secondary growth phase permits linear growth while utilizing the maximum oxygen transfer capacity of the reactor. When the desired biomass concentration is reached, PHB accumulation can conveniently be induced by nitrogen limitation. The PHB accumulation phase (phase 3) requires less oxygen, hence, carbon limitation is no longer necessary and pH-static feeding can be reinstated.

Fig. 3 illustrates the implementation of this triphasic strategy. In all fermentations, the first phase is characterized by exponential growth up to 13 g L^{-1} CDW. During phase 2, a constant feed rate was applied to keep the DO at 30% and enable linear growth. This was ended at three different biomass concentrations: 53.0, 58.5, and 66.4 g L^{-1} CDW. At that point, the PHB content amounted to only 10.7%, 28.0%, and 22.0%, respectively, confirming successful extension of the growth phase. In addition, the biomass productivity during this linear growth was found to exceed 2.0 g L^{-1} h^{-1} CDW, which corresponds to the growth rate at the end of the exponential growth phase and is among the highest results for *C. necator* (Koller, 2018). Subsequently, in phase 3, PHB was accumulated. The PHB content increased up to 70.0%, 69.0%, and 72.1%, reaching a final PHB titer of 62.6, 73.1, and 80.6 g L^{-1} PHB, respectively. From this, it is clear that an extension of the growth phase effectively leads to an increased PHB titer. In terms of productivity, a small decrease was observed for higher biomass concentrations during PHB accumulation, leading to an overall productivity of 0.83, 0.75, and 0.69 g L^{-1} h^{-1} PHB, respectively. This is in line with previous research on high cell density PHB fermentations, reporting reduced PHB productivity at higher biomass concentrations (Mozumder et al., 2014b). Conclusively, although PHB productivity was not significantly increased, the linear extension of the growth phase was proven an effective strategy to obtain a high-cell density culture on acetic acid, in turn resulting in an increased final PHB titer.

3.2.3. Intensification through a repeated fed-batch strategy

Next, to further increase the productivity of the complete process, a repeated fed-batch strategy was established. After each fed-batch fermentation, 0.5% v/v of the broth was retained in the fermenter to serve as inoculum for the next. This has the benefit of avoiding the downtime required for cleaning, filling, and sterilization of the bioreactor, while also eliminating the seed train, thereby increasing the overall volumetric productivity. In Fig. 4, the results of three consecutive fed-batch fermentations, each with an extension of the growth phase to a biomass concentration of around 50 g L^{-1} CDW, are presented.

The first fed-batch fermentation resulted in the production of 82.4 g L^{-1} CDW containing 69% PHB in 82 h, of which 0.6 g L^{-1} CDW was retained for the second fermentation producing 81.5 g L^{-1} CDW containing 70% PHB in 68 h. Similarly, 0.4 g L^{-1} CDW was retained for the third fermentation producing 79 g L^{-1} CDW containing 74% PHB in 63 h. The culture thus maintained its high metabolic activity throughout

three cycles producing a total amount of 172 g L^{-1} PHB in 213 h. Interestingly, the overall productivity increased over the individual batches: from 0.69 in the first to 0.84 and 0.93 g L^{-1} h^{-1} PHB in the second and third fed-batch fermentation, respectively. This was attributed to two effects. On the one hand, the growth phase was more efficient when using a repeated fed-batch strategy, since the lag phase caused by transferring the seed from the flask to the fermenter was avoided. On the other hand, the specific productivity during the PHB accumulation phase increased throughout the consecutive batches. The latter effect is in line with previous research on other carbon sources describing a repeated batch operation for more efficient PHB synthesis. As such, Khanna and Srivastava (2005) reported an increase in productivity from 0.15 to 0.42 g L^{-1} h^{-1} PHB by *C. necator* on fructose by harvesting and refilling 20% v/v of the culture broth. However, to the best of our knowledge, this is the first report describing a repeated fed-batch fermentation strategy for the production of PHB by *C. necator* from an organic acid, reaching industrially relevant PHB titers and productivities.

4. Conclusion

In this paper, the use of formic acid and acetic acid was investigated for the production of PHB with *C. necator*. A superior PHB production was observed on acetic acid, which could be attributed to the more efficient assimilation mechanism, as compared to formic acid. By improving the process stability with the use of NH_4-acetate as the nitrogen source, extending the biomass growth with a linear growth phase, and the implementation of a repeated fed-batch strategy, the PHB titer and productivity on acetic acid were substantially increased to 58.5 g L^{-1} PHB and 0.93 g L^{-1} h^{-1} PHB. To further pave the way for industrial PHB production from CO_2-derived organic acids, follow-up research could include strain engineering to develop micro-organisms capable of more efficient formic acid assimilation, as well as validation of the developed fermentation strategy in a representative, industrial setting.

CRediT authorship contribution statement

Elodie Vlaeminck: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Koen Quataert:** Formal analysis, Investigation, Writing – review & editing. **Evelien Uitterhaegen:** Writing – review & editing. **Karel De Winter:** Conceptualization, Project administration, Writing – review & editing. **Wim K. Soetaert:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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