Targeted poly(3-hydroxybutyrate-co-3-hydroxyvalerate) bioplastic production from carbon dioxide


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G R A P H I C A L A B S T R A C T

ARTICLE INFO

Keywords:
Gas fermentation
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
Carbon capture and utilization
Modelling

A microbial production process was developed to convert CO₂ and valeric acid into tailored poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) bioplastics. The aim was to understand microbial PHBV production in mixotrophic conditions and to control the monomer distribution in the polymer. Continuous sparging of CO₂ with pulse and pH-stat feeding of valeric acid were evaluated to produce PHBV copolymers with predefined properties. The desired random monomer distribution was obtained by limiting the valeric acid concentration (below 1 g L⁻¹). ¹H-NMR, ¹³C-NMR and chromatographic analysis of the PHBV copolymer confirmed both the monomer distribution and the 3-hydroxyvalerate (3HV) fraction in the produced PHBV. A physical-based model was developed for mixotrophic PHBV production, which was calibrated and validated with independent experimental datasets. To produce PHBV with a predefined 3HV fraction, an operating diagram was constructed. This tool was able to predict the 3HV fraction with a very good accuracy (2% deviation).

1. Introduction

The 2030 framework for climate and energy policies contains a binding target to cut greenhouse gas emissions in the EU by at least 40% below 1990 levels by 2030 and has the ambition to further reduce them by 80–95% by 2050 (Delbeke and Vis, 2016). As theoretical limits of efficiency are being reached and process-related emissions are sometimes inevitable, there is an urgent need to develop efficient carbon capture systems (Pachauri and Meyer, 2014). In the past, most research focused on the capture and storage of CO₂, also referred to as...
Carbon Capture and Storage (CCS). Alternatively, CO2 could be recognized as a valuable resource, which can be utilized for the production of carbon-based chemicals with current or future demand (Liu et al., 2015). This is known as carbon capture and utilization (CCU) and can provide much needed additional capacity in the move towards a low-carbon economy (Cheah et al., 2016).

Producing plastics from CO2 instead of oil constitutes an interesting case for CCU. Polyhydroxyalkanoate (PHA) is a class of biodegradable bioplastics (microbial polyesters) produced from renewable resources (Doi, 1991). A two-phase fermentation process is typically applied, comprising biomass growth, followed by PHA accumulation under nutrient-limiting conditions (Johnson et al., 2010; Grousseau et al., 2014). PHAs can be produced by various prokaryotic species, the most widely studied one being Cupriavidus necator (formerly referred to as Ralstonia eutropha, Alcaligenes eutrophus and Wautersia eutropha). C. necator has the capacity to grow and accumulate PHA autotrophically; using CO2 as the sole carbon source and H2 as energy source (Tanaka et al., 1995; Ishizaki and Tanaka, 1991). Deploying C. necator for PHA production can thus bridge CCU and bioplastic production (Kumar et al., 2017). So far, most experimental work on PHA production from CO2 has been conducted in view of optimizing the production of the homopolymer poly(3-hydroxybutyrate) (PHB) (Mozumder et al., 2015; Garcia-Gonzalez et al., 2015; Ishizaki and Tanaka, 1991).

Despite the interesting properties of PHB, its use as commodity plastic is hampered by its stiffness, brittleness and low impact strength. These physical properties can be improved by the inclusion of other monomers in the polymer (Kachrimanidou et al., 2014; Ray and Kalia, 2017). Indeed, copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) display greater ductility and toughness compared to PHB (Pan and Inoue, 2009). Producing targeted PHBV copolymers could thus extend the range of PHA bioplastic properties and applications. Targeted PHBV copolymer production can be achieved by altering (i) the monomer distribution (e.g. block copolymers or random copolymers) and (ii) the comonomer fractions in the PHA copolymer (Wang et al., 2001; McChalicher and Slience, 2007).

Copolymer production with organic substrates is well known. However, very few reports investigated mixotrophic PHA production in which CO2 is supplied in combination with an organic co-substrate (Park et al., 2014; Volova and Kalacheva, 2005; Volova et al., 2008; Volova et al., 2013). The upper part of Table 1 summarizes the state-of-the-art on mixotrophic or heterotrophic PHA copolymer production comprising 3HB and 3HV monomers, both in therm of experimental and modelling efforts. Table 1 indicates that valeric acid is the commonly investigated precursor of 3HV, while CO2 is the precursor for 3HB in mixotrophic conditions. Park et al. (2014), Volova and Kalacheva (2005), Volova et al. (2008) and Volova et al. (2013) exclusively applied pulse feeding the various organic co-substrates to evaluate their effect on the PHA production and composition. Mixotrophic PHA copolymer production, however, comprises a number of process steps in which a lot of process variables and other influencing factors are involved (Penloglou et al., 2012).

Modelling and simulation are very useful tools to understand the dynamic process behavior, the underlying mechanisms and to develop control strategies for maximizing PHA production (Novak et al., 2015). The production of PHA copolymers consisting of 3HB and 3HV monomers from exclusively organic substrates was modelled by Spoljaric et al. (2013) and Koller et al. (Dec 2006) (Table 1). Both studies assumed no influence of the consumption of one substrate to the consumption rate of the other, while the results of Park et al. (2014) indicated that such influences do take place between CO2 and valeric acid.

Table 1

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Growth phase</th>
<th>Accumulation phase</th>
<th>FAE type</th>
<th>Reactor type and operation</th>
<th>Modelling efforts</th>
<th>Reference</th>
</tr>
</thead>
</table>

Table 1 State-of-the-art of experimental and modelling efforts on PHBV production, deploying either mixotrophic conditions, or multi-substrate heterotrophic conditions. FAME: fatty acids methyl esters, 3HB: 3-hydroxybutyrate, 3HV: 3-hydroxyvalerate, 4HB: 4-hydroxybutyrate, 3HHx: 3-hydroxyhexanoate, 3HHp: 3-hydroxyheptanoate, 3HO: 3-hydroxyoctanoate, VA: valeric acid, PHBV: poly(3-hydroxybutyrate-co-3-hydroxyvalerate).
acid during mixotrophic fermentation. However, up until now no model exists for mixotrophic PHA copolymer production, which requires inclusion of such substrate interaction. This, together with the improved properties of PHBV compared to PHB, prompted us to focus on experimentation and modelling of substrate interaction and random PHBV production with alterable 3HV content, which can be set according to the envisaged applications.

This work is thus the first to study and model a mixotrophic production process for the production of PHBV copolymers with predefined composition. Experiments consisted of a first stage of cell mass growth using glucose as substrate, followed by PHBV copolymer production using CO₂ and valeric acid as carbon sources (Fig. 1). A pH-stat strategy was applied for the first time throughout the whole mixotrophic PHBV copolymer production phase. The developed model was calibrated and validated using data from independent experiments conducted in this study.¹H-NMR, ¹³C-NMR and chromatographic analyses of the PHBV copolymers were performed to assess whether the monomer distribution and 3HV fraction of PHBV were correctly predicted by the model.

2. Materials and methods

2.1. Experimental set-up

2.1.1. Organism and inoculum

C. necator, strain DSM 545 (Leibniz-Institut, DSMZ GmbH, Germany) was deployed as microorganism. Stock cultures were stored at −20 °C in 2 mL cryovials containing 0.5 mL glycerol (85%, Merck, Germany) and 1 mL of a late exponential-phase liquid culture in Lennox broth (LB) medium (Invitrogen, Life Technologies Europe B.V., Belgium). These stock cultures were used to inoculate preculture 1 by transferring 200 μL to 5 mL of LB-medium in 15 mL test-tubes. The preculture was cultivated in an orbital shaker (Innova 42, Eppendorf, USA) for 24 h at 30 °C and 200 rpm. Subsequently, 2 mL of the strain was sub-cultured during 24 h at 30 °C and 180 rpm in 100 mL of preculture 2 seeding medium in 500 mL baffled flasks. Finally, the seed culture was used to inoculate the bioreactor (12.5% v/v inoculum). Compositions of the culture and fermentation media are specified in Mozumder et al. (2014).

2.1.2. Bioreactor set-up and control

A 7 L, double jacked, lab-scale bioreactor unit with EZ-control system (Applikon Biotechnology, the Netherlands) for on-line monitoring and controlling of the stirring speed, dissolved oxygen (DO), foam formation, pH and temperature was used. Foam formation was measured through a level contact (conductivity) sensor and was controlled by the addition of 30% antifoam C emulsion (Sigma–Aldrich Chemie, GmbH, Germany). The process temperature was measured by a platinum resistance thermometer sensor (PT 100) and kept constant at 30 °C. The head space pressure of the bioreactor was controlled at 6.80 by adding acid (2 M H₂SO₄, 97%, Merck, Germany) or base (NH₄OH 28.0% NH₃ basis, Sigma–Aldrich Chemie GmbH, Germany). The ammonium concentration was maintained between 0.60 and 0.71 g N L⁻¹. Glucose (650 g L⁻¹, Merck, Germany) was fed exponentially the first 10 h, followed by a feeding regime based on alkali-addition to control the glucose concentration at 12 g L⁻¹ (Mozumder et al., 2014).

2.1.3. Operating conditions of the heterotrophic phase

The first phase was carried out according to Garcia-Gonzalez et al. (2015). The temperature was set to 30 °C, the agitation speed 950 rpm and the pressure 1 bar. The DO concentration was maintained around 55% of air saturation during heterotrophic growth using a cascade control strategy consisting of the agitation speed (950 rpm), air and/or oxygen flow. These relatively high DO levels were chosen to ensure that heterotrophic growth was not limited by the O₂ concentration. The pH was controlled at 6.80 by adding acid (2 M H₂SO₄, 95–97%, Merck, Germany) or base (NH₄OH 28.0–30.0% NH₃ basis, Sigma–Aldrich Chemie GmbH, Germany). The ammonium concentration was maintained between 0.60 and 0.71 g N L⁻¹. Glucose (650 g L⁻¹, Merck, Germany) was fed exponentially the first 10 h, followed by a feeding regime based on alkali-addition to control the glucose concentration at 12 g L⁻¹ (Mozumder et al., 2014).

2.1.4. Operating conditions of the mixotrophic phase

The mixotrophic phase was initiated once the biomass concentration reached approximately 15 g L⁻¹ by ceasing glucose addition and replacing NH₄OH by NaOH for pH control. Once the nitrogen concentration dropped below 100 mg L⁻¹ due to consumption, the agitation speed was increased to 1200 rpm and CO₂ (industrial X50S, Air Products, Belgium), H₂ (technical X50S, Air Products, Belgium) and O₂ (industrial X50S, Air Products, Belgium) were continuously sparged into the bioreactor, keeping a constant gas composition in the headspace of H₂:O₂:CO₂ = 84:2.8:13.2 vol% at 80 mbar overpressure (Garcia-Gonzalez et al., 2015). Under such conditions, nitrogen and oxygen became limited, triggering biopolymer synthesis. Two valeric acid feeding strategies were tested: (i) pulse-feeding to study mixotrophic conditions and perform model calibration and (ii) semi-con- tinuous addition by a pH-stat to produce targeted PHBV copolysters and perform model validation. Samples were taken at regular time intervals for analysis.

Pulse-feeding was performed during a first experiment by spiking valeric acid (99%, Sigma–Aldrich Chemie, GmbH, Germany) into the
bioreactor four times during the biopolymer production phase. Three pulses at 2, 24 and 47 h were given to reach a valeric acid concentration of 1.5 g L\(^{-1}\). The fourth pulse was added after 68 h in the biopolymer production phase, envisaging a valeric acid concentration of 0.5 g L\(^{-1}\) and ensuring all valeric acid was consumed at the end of the fermentation run. 2 M H\(_2\)SO\(_4\) was used for pH control.

Semi-continuous addition of valeric acid through a pH-stat approach (Huschner et al., 2015) was studied in a second experiment, using 920.7 g L\(^{-1}\) valeric acid for pH control. At the start of the mixotrophic phase, 0.741 mL valeric acid was added to reach a medium concentration of 0.239 g L\(^{-1}\) valeric acid and O\(_2\). The pH was corrected to 6.80 with NaOH. Consumption of valeric acid for 3HV production initialized the pH-stat cycle: when the pH of the mineral medium increased upon valeric acid consumption, extra valeric acid was added to maintain the pH at optimum level. By doing so, the pH and valeric acid inflow rate were aimed constant.

2.1.5. Analytical procedures

The concentrations of glucose, ammonium-nitrogen (NH\(_4\)-N), cell dry weight and 3HB concentrations were determined as in Mozumder et al. (2014). To determine the 3HV concentrations, a standard of PHBV (Sigma-Aldrich Chemie, GmbH, Germany) was included. The stoichiometry and kinetics for these conversions are summarized in Table 2 and Table 3, respectively. The stoichiometry for 3HB production on CO\(_2\), O\(_2\) and H\(_2\) was reported by Ishizaki and Tanaka (1991):

<table>
<thead>
<tr>
<th>Conversion</th>
<th>Reaction rate</th>
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<tbody>
<tr>
<td>1</td>
<td>( \rho_{3HB} = \rho_{3HB} \cdot X ), with ( \rho_{3HB} = \rho_{3HB}^{\text{max}} \cdot \frac{\rho_{2}}{K_{O_2} + \rho_{2}} \cdot \frac{\rho_{O_2}}{K_{O_2} + \rho_{O_2}} \cdot \frac{\rho_{CO_2} \cdot \rho_{CO_2} + \rho_{C_{2}H_{4}}}{} \cdot \frac{1}{K_{PIN} \cdot \rho_{PIN}} )</td>
</tr>
<tr>
<td>2</td>
<td>( \rho_{3HV} = \rho_{3HV} \cdot X ), with ( \rho_{3HV} = \rho_{3HV}^{\text{max}} \cdot \frac{\rho_{2}}{K_{O_2} + \rho_{2}} \cdot \frac{\rho_{O_2}}{K_{O_2} + \rho_{O_2}} \cdot \frac{\rho_{Val} \cdot K_{PIN} \cdot N \cdot K_{PIN}}{K_{PIN} + \rho_{PIN}} )</td>
</tr>
<tr>
<td>3</td>
<td>( \rho_{m,3HB} = m_{3HB} \cdot X ), with ( m_{3HB} = m_{3HB}^{\text{max}} \cdot \frac{\rho_{3HB}}{K_{3HB} + \rho_{3HB}} \cdot \frac{\rho_{3HB}}{K_{3HB} + \rho_{3HB}} )</td>
</tr>
<tr>
<td>4</td>
<td>( \rho_{m,3HV} = m_{3HV} \cdot X ), with ( m_{3HV} = m_{3HV}^{\text{max}} \cdot \frac{\rho_{3HV}}{K_{3HB} + \rho_{3HV}} \cdot \frac{\rho_{3HV}}{K_{3HV} + \rho_{3HV}} )</td>
</tr>
</tbody>
</table>
Table 4  Model parameter values from parameter estimation, calculations and assumptions (θopt).

<table>
<thead>
<tr>
<th>Estimated parameter</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \theta_{\text{opt}} )</td>
<td>0.01808</td>
<td>[g 3HB g X(^{-1}) l(^{-1})]</td>
</tr>
<tr>
<td>( \mu_{\text{max}} )</td>
<td>0.1243</td>
<td>[g 3HV g X(^{-1}) l(^{-1})]</td>
</tr>
<tr>
<td>( \mu_{\text{min}} )</td>
<td>0.001123</td>
<td>[g 3HV g X(^{-1}) l(^{-1})]</td>
</tr>
<tr>
<td>( \theta_{\text{min}} )</td>
<td>0.0066361</td>
<td>[g 3HV g X(^{-1}) l(^{-1})]</td>
</tr>
<tr>
<td>( \theta_{\text{max}} )</td>
<td>20.092</td>
<td>[g 3HV g X(^{-1}) l(^{-1})]</td>
</tr>
<tr>
<td>( H_2 )</td>
<td>9.5758e-05</td>
<td>[g H(_2) l(^{-1})]</td>
</tr>
<tr>
<td>( O_2 )</td>
<td>0.002483</td>
<td>[g O(_2) l(^{-1})]</td>
</tr>
<tr>
<td>( CO_2 )</td>
<td>9.7086e-4</td>
<td>[g CO(_2) l(^{-1})]</td>
</tr>
<tr>
<td>( k_{\text{V}} )</td>
<td>9.0447</td>
<td>[g Val l(^{-1})]</td>
</tr>
<tr>
<td>( k_{\text{HV}} )</td>
<td>5.4288</td>
<td>[g 3HV l(^{-1})]</td>
</tr>
<tr>
<td>( k_{\text{HB}} )</td>
<td>7.5534</td>
<td>[g 3HB l(^{-1})]</td>
</tr>
<tr>
<td>( c )</td>
<td>99.0287</td>
<td>[ ]</td>
</tr>
<tr>
<td>( n )</td>
<td>1.0044</td>
<td>[ ]</td>
</tr>
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Calculated parameters | Value | Unit |
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>( Y_{\text{Val},\text{Val}} )</td>
<td>( \text{inf}_{\text{inf}} = 0.64 )</td>
<td>[g 3HV g Val(^{-1})]</td>
</tr>
<tr>
<td>( V_{\text{Val}} )</td>
<td>920.7</td>
<td>[g Val l(^{-1})]</td>
</tr>
<tr>
<td>( f )</td>
<td>0.65</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

Value-assumed parameters | Value | Unit |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( N )</td>
<td>0</td>
<td>[g N l(^{-1})]</td>
</tr>
</tbody>
</table>

\[
3\text{H}_2 + 12\text{O}_2 + 4\text{CO}_2 \rightarrow \text{C}_6\text{H}_4\text{O}_2 + 3\text{H}_2\text{O},
\]

where \( \text{C}_6\text{H}_4\text{O}_2 \) represents the elemental composition of a 3HB monomer. The production rate of 3HB (\( \rho_{3HB} \)) from Mozumder et al. (2015) was adapted for mixotrophic conditions. Limitation of the 3HB production rate by \( H_2 \) and \( CO_2 \) was modelled through Monod kinetics. For \( O_2 \), both limitation and inhibition were taken into account (through Haldane kinetics) as the \( O_2 \) concentration exceeded the inhibition constant from Tanaka et al. (1995). The inhibition of 3HB production in the presence of a nitrogen source was accounted for through a non-competitive inhibition equation (Spoljaric et al., 2013; Mozumder et al., 2014). The decrease of 3HB synthesis from \( CO_2 \) in the presence of non-limiting valeric acid concentrations, observed by Park et al. (2014) was accounted for through a generalized non-competitive inhibition equation (Kwon and Engler, 2005).

The conversion of valeric acid to a 3HV monomer was modelled in this work, using the generic conversion of organic substrate to a monomer from Akiyama et al. (2003). However, both experiments consistently indicated that one mole of valeric acid did not yield one mole 3HV. Only a fraction \( f \) of valeric acid was directly converted to 3HV. The remainder (1−\( f \)) was assumed to be oxidized with \( O_2 \) to \( CO_2 \) and \( H_2O \), in line with Spoljaric et al. (2013) (Table 1) and Akiyama et al. (2003). The overall conversion is given by Eq. (2):

\[
\text{C}_6\text{H}_{10}\text{O}_2 + \left( \frac{11}{2} - 6f \right) \text{O}_2 \rightarrow f \text{C}_6\text{H}_4\text{O}_2 + (5-4f)\text{H}_2\text{O} + (5-f)\text{CO}_2.
\]
concentrations were quasi constant during the whole process.

Model validation was performed with independent data from a distinct experiment by calculating validation errors, i.e. the absolute deviation of new experimental data for the valeric acid, 3HV and 3HB concentrations from values predicted with the calibrated model:

\[ |y_i(t) - \chi_i(t, \theta)| \]

2.2.4. Calculation of process performance parameters

The residual cell concentration (RCC) is defined as:

\[ \text{RCC} = C_{\text{DW,3HB}} - C_{\text{3HV}} \]

where \( C_{\text{DW,3HB}} \) and \( C_{\text{3HV}} \) represent the cell dry weight and monomer concentrations respectively. The fraction of comonomer 3-hydroxyvalerate at time \( t \) is calculated as:

\[ F_t = \frac{C_{\text{3HV}}(t)}{C_{\text{3HV}}(t) + C_{\text{3HB}}(t)} \] (10)

Similarly, the PHBV content \( F_{\text{PHBV}} \) (polymer fraction in the microorganisms) was calculated from the PHBV concentration and the residual biomass concentration \( X \):

\[ F_{\text{PHBV}} = \frac{3HV + 3HB}{3HV + 3HB + X} \]

The synthesis rate of PHBV (productivity) \( -\frac{\text{gPHBV}}{L.h} \) was calculated from the final PHBV concentration \( (\text{PHBV}) [\text{g PHBV L}^{-1}] \) and the duration of the process \( \Delta t \) [h]: \( \text{PHBV}/\Delta t \). The 3HB and 3HV synthesis rate were calculated in a similar form from the monomer concentrations and process duration.

The experimental yield of 3HV over valeric acid \( [\text{g 3HV g Val}^{-1}] \) was calculated from the accumulated 3HV concentration \( 3HV \ [\text{g 3HV L}^{-1}] \) after consumption of valeric acid \( \text{Val} \ [\text{g Val L}^{-1}] \). The theoretical yield of 3HV over valeric acid \( [\text{g 3HV g Val}^{-1}] \) is 0.98 (mass based), obtained for \( f = 1 \) in 100f/102 from Table 2. The experimentally measured fraction \( f \) from the maximal 3HV yield over valeric acid was calculated as the ratio of the experimental 3HV yield over valeric acid and the maximal 3HV yield over valeric acid.

The dyad sequence distribution \( D \) and degree of randomness \( R \) to analyse the polymers microstructure were calculated according to Zagar et al. (2006).

3. Results and discussion

Two heterotrophic-mixotrophic fermentation experiments were conducted for PHBV production from CO2 and valeric acid. Data from a first experiment with valeric acid pulses and CO2 sparging were used to establish the stoichiometric conversion from valeric acid to 3HV, to calibrate the model and assess substrate interaction. In a second experiment, a pH-stat mediated valeric acid addition strategy was designed to produce predefined PHBV copolyesters in mixotrophic conditions. Data from this experiment were used for model validation.

3.1. Pulse-feeding valeric acid in mixotrophic conditions

The experimental results for PHBV synthesis, show a total 3HB concentration of 14.4 g L\(^{-1}\) after 70 h in the mixotrophic phase (Fig. 2), while the 3HV concentration amounted to 1.5 g L\(^{-1}\) (Fig. 2), corresponding to a PHBV content of 51.5% \( [X=15 \text{ g L}^{-1}] \).

3.1.1. 3HV conversion stoichiometry

The experimental results for PHBV synthesis, show a total 3HB concentration of 14.4 g L\(^{-1}\) after 70 h in the mixotrophic phase (Fig. 2), while the 3HV concentration amounted to 1.5 g L\(^{-1}\) (Fig. 2), corresponding to a PHBV content of 51.5% \( [X=15 \text{ g L}^{-1}] \).
\[ \text{C}_3\text{H}_6\text{O}_2 + 2.6\text{O}_2 \rightarrow 0.65\text{C}_2\text{H}_4\text{O}_2 + 2.4\text{H}_2 \text{O} + 1.75\text{CO}_2. \] 

The obtained molar 3HV yield over valeric acid \((f = 0.65)\) is approximately twice as high as those obtained by Gahlawat and Soni (2017), which were 0.28 mol 3HV mol Val\(^{-1}\) and 0.30 mol 3HV mol Val\(^{-1}\) for 2 g L\(^{-1}\) and 4 g L\(^{-1}\) valeric acid respectively. We assume this can be explained by the more equal substrate preference between the two organic substrates glycerol and valeric acid in Gahlawat and Soni (2017). Compared to CO2, which is converted through the energy-demanding Calvin-Benson-Bassham (CBB) cycle, valeric acid can be regarded as a more favorable substrate, as will be motivated further in this section. Therefore, the valeric acid yield towards 3HV would be higher in mixotrophic conditions. Results of (Park et al., 2014) support this reasoning. Indeed, the obtained molar 3HV yield over valeric acid in mixotrophic conditions varies between 0.53 mol 3HV mol Val\(^{-1}\) and 0.57 mol 3HV mol Val\(^{-1}\), which is closer to the values obtained in our study. The deviation between our findings and those of Park et al. (2014) may be attributed to other factors, such as a different gas composition. Park et al. (2014) applied H\(_2\)O:CO\(_2\) = 77.78:11.11:11.11 vol\% as opposed to (H\(_2\)O:CO\(_2\) = 84:2.8:13.2 vol\%) applied in this study, which also not lays in the explosion range of hydrogen gas.

### 3.1.3. Substrate interaction between CO\(_2\) and valeric acid

The interaction between both substrates was assessed by focusing on the predicted and experimental 3HB and 3HV concentrations just before and after the first pulse in Fig. 2. Time zero denotes the start of the mixotrophic phase.

\[ \text{3HB (predicted)} \quad \text{3HB (experimental)} \quad \text{3HV (predicted)} \quad \text{3HV (experimental)} \quad \text{Valeric acid (predicted)} \quad \text{Valeric acid (experimental)} \]

![Fig. 3. Detail of the experimental and simulated concentrations of valeric acid and the monomers 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) just before and after the first pulse in Fig. 2. Time zero denotes the start of the mixotrophic phase.](image)

**3.1.2. Model calibration**

The estimated parameters obtained upon minimizing the objective function \((\theta^{opt})\) are represented in Table 4. The coefficients of determination, \(R^2\), were determined to compare the calibrated model output with the experimental observations. \(R^2\) was 0.8336 for the valeric acid concentration, 0.4875 for the 3HV concentration and 0.9360 for the 3HB concentration. The predicted PHBV concentration is the sum of its monomer concentrations (3HB + 3HV) and had a \(R^2\) of 0.9385. The good agreement between the simulation results and experimental measurements for the valeric acid concentrations, 3HB and PHBV concentrations is also apparent in Fig. 2. Only for the last four 3HV concentrations a small deviation of the predictions was observed. Overall, the model captured most essential microbial processes and can reasonably explain the experimental observations.

**Table 4**, that its contribution was not visible. Immediately after the pulse, the 3HB production rate decreased dramatically and the consumption rate of 3HB for maintenance became visible (as a slightly negative 3HB accumulation rate). As valeric acid is converted, the 3HV concentration increased.

Although this is not explicitly mentioned, the results of Park et al. (2014) also indicated the existence of a critical valeric acid concentration, which in their study amounted to 0.46 g L\(^{-1}\) valeric acid. This value was therefore denoted as the ‘critical valeric acid concentration’. Deriving the critical valeric acid concentration after model calibration guaranteed that all data was taken into account in its calculation. Also, the experimental measurements were insufficient to adequately annotate a critical valeric acid concentration, due to the very fast valeric acid conversion. The critical valeric acid concentration was identical for the other pulses (Supplementary Information). For the subsequent experiment, the valeric acid concentration was therefore kept below 1 g L\(^{-1}\) to obtain random PHBV.

Although this is not explicitly mentioned, the results of Park et al. (2014) also indicated the existence of a critical valeric acid concentration, which in their study amounted to 0.46 g L\(^{-1}\) valeric acid. This is about three times less than that obtained in this study. This difference may be attributed to the different operating conditions.

Our findings and those of Park et al. (2014), thus indicate that autotrophic and heterotrophic monomer synthesis pathways can coexist, but only if heterotrophic substrate is limiting. This is in accordance with Schwartz et al. (2009) and Shimizu et al. (2015), who evidenced the
coexistence of both the autotrophic and heterotrophic pathways during bacterial growth and PHA accumulation. For the energy housekeeping of \textit{C. necator} in heterotrophic conditions, it is beneficial to repress the \textit{ccb} genes involved in CO$_2$ fixation by the energy intensive CBB cycle. However, partial derepression occurs on some substrates (e.g. fructose) to convert CO$_2$ from oxidative decarboxylation into 3HB monomers (Shimizu et al., 2015), in order to circumvent adverse effects of CO$_2$ on cell functioning. In our study, we assume that the supplied CO$_2$ was converted to 3HB, rather than CO$_2$ from decarboxylation of valeric acid intermediates, because the presence of CO$_2$ thermodynamically hampers such a decarboxylation.

The repression and derepression of the \textit{ccb} genes is achieved through the CbbR protein in \textit{C. necator} (CbbR$^{\text{RE}}$), which is modulated by metabolites signaling the nutritional state of the cell to the \textit{cbb} system (Esparza et al., 2015). Above the critical valeric acid concentration, CO$_2$ fixation seems to be entirely repressed, while at

Fig. 4. Concentrations of dry cell weight, residual biomass, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) during the mixotrophic phase of the experiment with pH-stat addition of valeric acid under constant sparging of CO$_2$, H$_2$ and O$_2$. Time zero denotes the start of the mixotrophic phase.

Fig. 5. Model validation with independent data for the prediction of 3-hydroxybutyrate, 3-hydroxyvalerate and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and the valeric acid concentrations. Time zero denotes the start of the mixotrophic phase.
limiting valeric acid levels, it is only partially repressed. Given this partial repressing, we suggest that a metabolite of the valeric acid-to-3HV pathway signals the cell to mainly utilize valeric acid, while CO2 fixation to 3HB remains partly derepressed at low levels of this valeric acid-derived metabolite.

3.2. Semi-continuous valeric acid feeding in mixotrophic conditions

3.2.1. Process performance

The semi-continuous valeric acid feeding rate was 0.003 L h⁻¹ with a valeric acid concentration of 0.75 ± 0.47 g L⁻¹. Online monitoring of the pH and cumulative addition of valeric acid and NaOH over time is displayed in the Supplementary Information. The evolution of the dry cell weight and monomers is represented in Fig. 4. In this experiment, the total 3HB concentration reached a maximal level of 7.0 g L⁻¹, while 3HV amounted to 17.7 g L⁻¹ within a 28 h accumulation phase (Fig. 4). So, a total PHBV concentration of 24.7 g L⁻¹ was achieved. The simultaneous production of both monomers in Fig. 4 is indicative for the production of random PHBV. The measured residual biomass concentration was initially 13.9 g L⁻¹, but decreased to 6.9 g L⁻¹, possibly due to larger valeric acid additions during the pH-stat, causing inhibitory effects and cell lysis (Khanna and Srivastava, 2007). The PHBV content at the end of the experiment was 78% (24.66 g PHBV L⁻¹/31.49 g (PHBV + X) L⁻¹), with a 3HV fraction in PHBV of 0.72 (17.7 g 3HV L⁻¹/24.66 g PHBV L⁻¹). The obtained experimental 3HV yield over valeric acid was 0.69 (17.7 g 3HV L⁻¹/24.45 g Val L⁻¹), resulting in a value of 0.70 for the experimentally measured fraction f from the maximal 3HV yield over valeric acid. This is close to the 0.65 previously determined, confirming the annotated stoichiometry in Eq. (11).

Garcia-Gonzalez et al. (2015) used identical autotrophic conditions (H₂O₂:CO₂ = 84:2.8:13.2 vol%) to produce pure PHB and reached a polymer concentration of 28 g L⁻¹, which is comparable to the 24.7 g L⁻¹ obtained in this work. Nevertheless, the productivity from Garcia-Gonzalez et al. (2015) was 0.17 g L⁻¹ h⁻¹ and significantly lower than the productivity of 0.87 g L⁻¹ h⁻¹ obtained in this study. This is attributed to the higher accumulation rate of heterotrophic valeric acid. In the study of Park et al. (2014), both valeric acid and a gas mixture of H₂, O₂ and CO₂ (H₂:O₂:CO₂ = 77.78:11.11:11.11 vol%) were used during a six day accumulation phase. The final PHBV concentration was 1.07 g L⁻¹. The corresponding productivity of 0.007 g L⁻¹ h⁻¹ was significantly lower than the 0.87 g L⁻¹ h⁻¹ obtained in this experiment. This may be due to a lower biomass concentration after the biomass growth phase, although that concentration was not reported.

The 3HV synthesis rate was 0.63 g L⁻¹ h⁻¹, while the 3HB synthesis rate was 0.25 g L⁻¹ h⁻¹. This contradicts with a reported 39% slower reactivity of the polymerase enzyme phaC towards R-3HV-CoA (relative to R-3HB-CoA), which are the last metabolites in the pathway to PHBV monomers (Zhang et al., Jul 2001). However, macroscopic and microscopic phenomena may explain this discrepancy. On a macroscopic level, valeric acid already is in the liquid phase of the medium, while the gaseous substrate experiences an additional resistance for mass transfer to the liquid phase. This favors a faster conversion of valeric acid into R-3HV-CoA and subsequent addition of a 3HV monomer to the growing PHBV copolyester. On a microscopic level, CO₂ has to undergo much more enzymatic conversion steps in the CBB cycle compared to valeric acid, motivating a faster 3HV synthesis. Indeed, the reactivity of phaC only relates to R-3HV-CoA and R-3HB-CoA, not taking into account upstream processes leading to these metabolites. Compared to Park et al. (2014), this work achieved a 525 times higher 3HV synthesis rate and a 26 times higher 3HB synthesis rate. The significantly higher 3HV synthesis rate can be attributed to the constant presence of valeric acid by the pH-stat approach. Indeed, Park et al. (2014) only provided valeric acid once or twice at the beginning of the accumulation phase. The obtained higher 3HB synthesis rate in this work may indicate that process conditions from Garcia-Gonzalez et al. (2015) are more optimal than those of Park et al. (2014).

3.2.2. Model validation

For a series of substrate, monomer and polymer concentrations, the validation errors (absolute deviations) between the new experimental data and the model outputs are represented in Fig. 5. The predicted 3HB, 3HV and PHBV concentrations are close to the experimental data (Fig. 5). The 3HV error/measurement ratio was on average 10%, with a maximum of 31% for the fourth data point, while the 3HV error/measurement ratio was on average 21%, with a maximum of 51% for the third data point. For PHBV, the PHBV error/measurement ratio was 14% on average, with a maximum of 37% for the third data point. For the valeric acid concentration, the validation errors were low at the beginning of phase two, but larger at the end. The valeric acid error/measurement ratio was on average 134%, with a maximum of 321% for the fourth data point. These larger biases are caused by larger variations in valeric acid concentrations, probably due to sub-optimal pH-stat control (Supplementary Information) and the observed interference of cell lysis prompted by temporary higher valeric acid concentrations on HPLC analysis. However, as the goal of the model was to predict the production of PHBV and its monomers, rather than the valeric acid concentration, the calibrated model is valid for its purpose and was used to set up an operating diagram for tailored synthesis of PHBV.

3.2.3. Operating diagram for targeted PHBV production

The calibrated model was applied to predict the 3HV fraction and PHBV content evolution over time for various valeric acid inflow rates and a residual biomass concentration of 13.9 g L⁻¹. The results were summarized in an operating diagram, displayed in Fig. 6. When applied to the second experiment (valeric acid inflow rate of 0.003 g L⁻¹ for 28 h mixotrophic fermentation), a 3HV fraction of F₃HV = 0.70 was predicted, matching the experimentally measured value of 0.72 very accurately. The operating diagram also indicated a PHBV content F₃HV = 0.61 of (61%), while the experimentally obtained one was 78.30%. The difference is attributed to the decrease of residual biomass over time (from 13.9 g L⁻¹ to 6.8 g L⁻¹ in Fig. 4), while simulations were conducted assuming a constant concentration of initial residual biomass. The PHBV content calculated with the final residual biomass concentration was 60%, which is close to its prediction. The operating diagram from Fig. 6 can thus be applied to produce PHBV copolyesters with a predefined composition. It demonstrates that low 3HV fractions (<0.50) are achieved for low valeric acid inflow rates.
(≤1.5 mL h⁻¹). Higher valeric acid inflow rates (between 3 and 4 mL h⁻¹) can also result in PHBV with 3HV fractions lower than 0.50, but only in the very beginning of the mixotrophic phase. However, the predicted PHBV content is only 10–20% in such case. With inflow rates of e.g. 1.5 mL h⁻¹, a PHBV copolymer with 50% 3HV is obtained after 30 h. From then on, the biopolymer composition does not change anymore, yet the PHBV contents still increase over time. The operating diagram can then be used to indicate how long the process has to be run to achieve a certain PHBV content. For instance, a PHBV content of 60% with 50% 3HV is achieved after 50 h. The latter demonstrates how the mixotrophic pH-stat production has to be run for targeted PHBV copolymer synthesis.

3.2.4. NMR spectroscopic validation of targeted PHBV production

The annotated ¹H-NMR and ¹³C-NMR spectra of PHBV obtained after the heterotrophic-mixotrophic experiment with a pH-stat set-up are provided in the Supplementary Information, together with a table containing the chemical shift, sequence and relative peak intensities of specific peaks. Using these data, the dyad sequence distribution for the final PHBV sample was calculated to be D = 0.86, while the degree of randomness was R = 0.85. Since D and R are close to 1, it can be concluded that the PHBV produced in this study exhibited a random sequence, as suggested by the simultaneous production of 3HB and 3HV in Fig. 4. The pH-stat set-up was indeed able to produce random PHBV. Moreover, the 3HV fraction determined by HPLC (0.72) was also confirmed by the measured 3HV fraction of 0.74 obtained through NMR analysis (converted from the mole based 3HV fraction of 0.64 in Supplementary Information, using molecular weight of 3HV).

3.3. Implications, limitations and future perspectives

This research shed light on several aspects of mixotrophic PHBV copolyester production and its modelling. Autotrophic 3HB production and heterotrophic 3HV production can coexist, however, regulation of both pathways in such conditions should be further elucidated. Nonetheless, this work demonstrates simultaneous valorization of CO₂ and organic residue streams to PHBV, the properties and applications of which can be further explored. The developed model can be used by others and can also be extended to model the production of other PHA copolymers. Limitations of this work are inherently coupled to gas fermentation in general, that is, low solubility of gases (mostly H₂) and limited productivity, which should be addressed by future research.

4. Conclusions

A mixotrophic fermentation process was demonstrated suitable for PHBV production, using CO₂, O₂ and H₂ as 3HB precursors and valeric acid as 3HV precursor. Below a critical valeric acid concentration of 1.14 g L⁻¹, 3HB and 3HV were simultaneously synthesized. A model for the production of both 3HB and 3HV monomers was set up, calibrated and validated. An operating diagram was constructed, from which the 3HV fraction of PHBV could be accurately predicted. This operating diagram can guide others to produce other specific PHB copolymers. The 3HV fraction and monomer distribution of PHBV were confirmed through NMR analysis.

Acknowledgements

The authors gratefully acknowledge prof. dr. ir. Christian Stevens and lab technicians of the SynBioC Research Group, Department of Sustainable Organic Chemistry and Technology Ghent University for performing NMR analysis of the PHA copolymers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at: http://dx.doi.org/10.1016/j.biotech.2017.10.081.

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