Biochemical Engineering Journal 114 (2016) 202–208

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Model-based process analysis of heterotrophic-autotrophic poly(3-hydroxybutyrate) (PHB) production

Md. Salatul Islam Mozumder[a,b,c,*], Linsey Garcia-Gonzalez[b], Heleen De Wever[b], Eveline I.P. Volcke[a]

[a] Ghent University, Department of Biosystems Engineering, Coupure Links 653, 9000 Gent, Belgium
[b] Flemish Institute for Technological Research (VITO), Business Unit Separation and Conversion Technology, Boeretang 200, 2400 Mol, Belgium
[c] Shahjalal University of Science and Technology, Department of Chemical Engineering and Polymer Science, Sylhet 3114, Bangladesh

ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form 20 June 2016
Accepted 8 July 2016
Available online 10 July 2016

Keywords:
Poly(3-hydroxybutyrate) (PHB)
Heterotrophic-autotrophic cultivation
CO_2
Mathematical modelling
Simulation

ABSTRACT

Heterotrophic-autotrophic poly(3-hydroxybutyrate) (PHB) production is a promising process to produce bioplastics from CO_2, which is more sustainable and safe compared to completely heterotrophic or completely autotrophic alternatives, respectively. In this study, pure culture heterotrophic-autotrophic PHB production was described through a mathematical model, which was validated on experimental datasets obtained with different organic substrates and applying different switching points from growth phase to PHB production phase. The mathematical model provided an accurate prediction of the dynamic behaviour of heterotrophic biomass growth and autotrophic PHB production. The model was subsequently applied for scenario analysis, to determine the optimal operation conditions in terms of O_2 and NH_4^+-N concentrations in the fermentation medium. The optimal scenario for maximal PHB production was 0.224 mg/L oxygen concentration at ammonium-nitrogen (NH_4^+-N) free condition.

1. Introduction

Poly(3-hydroxybutyrate) (PHB) is an intracellular storage material that is synthesized by a number of microorganisms and has become of considerable industrial interest and of environmental importance as a biodegradable and bio-based plastic. Although PHB is regarded as an effective substitute for conventional plastics, commercialization of this biopolymer is hampered by its high production cost compared to other (bio)polymers [1].

PHB is mostly produced through pure-culture fermentation, in which a cell growth phase under nutrient-sufficient conditions is followed by a PHB production phase triggered by applying nutrient (typically ammonium-nitrogen) limitation, making up a two-phase process. Most often heterotrophic conditions are applied during both phases, employing a wide variety of organic substrates, either pure substrates such as glucose, sucrose, starch, cellulose, or waste substrates such as molasses, whey, waste glycerol etc [2–5]. However, also autotrophic PHB production is possible by applying bacteria which use carbon dioxide (CO_2) as a carbon source and hydrogen (H_2) as an energy source [6–8]. Autotrophic PHB production is particularly interesting because it produces a valuable product and at the same time reduces the concentration of the greenhouse gas CO_2, in this way contributing to climate change mitigation. However, autotrophic PHB production is limited by the fact that the oxygen (O_2) concentration in the gas phase needs to be kept below the lower level of explosion, i.e. between 6 and 6.9% O_2 by volume in the gas mixture of CO_2, H_2, and O_2 [9]. The O_2 transfer rate becomes very low under this condition, which seriously hampers the cell growth because of O_2 limitation [10]. As a result, autotrophic–autotrophic PHB production, i.e. combining autotrophic growth (phase 1) and autotrophic PHB production (phase 2) cannot be realized in an economically viable way.

To overcome this limitation, a new cultivation method, namely heterotrophic-autotrophic PHB production, was proposed by Tanaka and Ishizaki, [10]. This method consists of heterotrophic growth on organic substrate (phase 1), maintaining nutrient-sufficient conditions, followed by autotrophic PHB production on CO_2, H_2, and O_2 while applying ammonium-nitrogen limitation (phase 2). In this way, a high PHB concentration can be realized under safe operating conditions.

Cupriavidus necator (formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus*, and *Wautersia eutropha*) is a well-known and well-studied hydrogen oxidizing bacterium that is able to grow

* Corresponding author at: Shahjalal University of Science and Technology, Department of Chemical Engineering and Polymer Science, Sylhet 3114, Bangladesh.
E-mail addresses: mdsalatulislam.mozumder@ugent.be, salatul-cep@sust.edu, msimozumder@gmail.com (Md.S.I. Mozumder).

http://dx.doi.org/10.1016/j.bej.2016.07.007
1369-703X/

Table 1
Stoichiometry for heterotrophic-autotrophic PHB production.

Component → Process ↓	Substrate (S)	Nutrient (NH_4^+)	Carbon dioxide (CO_2)	Hydrogen (H_2)	Oxygen (O_2)	Residual biomass (X)	Heterotrophic PHB (P_{het})	Autotrophic PHB (P_{aut})	Process rate
1. Biomass growth on substrate[a]	$-1/Y_{xS}$	$-1/Y_{xN}$			$-1/Y_{xO}$	1			$\mu_{xs}X$
2. Biomass growth on PHB[b]		$-1/Y_{xN}$			$-1/Y_{xO}$	1	$-1/Y_{xp}$		$\mu_{xp}X$
3. Heterotrophic PHB production[c]	$-1/Y_{pS}$				$-1/Y_{pO}$		1		$\mu_{ps_{het}}X$
4. Autotrophic PHB production[d]			$-1/Y_{pCO2}$	$-1/Y_{pH2}$	$-1/Y_{pO2}$			1	$\mu_{ps_{aut}}X$

[a] The parameters Y_{xS}, Y_{xN} and Y_{xO} are related to each other through the COD and N-balances.
[b] The parameters Y_{xp}, Y_{xN} and Y_{xO} are related to each other through the COD and N-balances.
[c] The parameters Y_{pS} and Y_{pO} are related to each other through the COD balance.
[d] The parameters Y_{pCO2}, Y_{pH2} and Y_{pO2} are related to each other and to the stoichiometric coefficient of H_2O (not explicitly taken up) through the C, H and O balances.

under both heterotrophic conditions, using organic carbon, and autotrophic conditions, on a gas mixture (CO_2, O_2 and H_2) [11]. In the presence of sufficient O_2 and ammonium-nitrogen (NH_4^+-N), *C. necator* ($CH_{1.74}O_{0.46}N_{0.19}$) grows on organic substrate ($C_xH_yO_z$) according to Eq (1).

$$C_xH_yO_z + O_2 + NH_4^+ \rightarrow CH_{1.74}O_{0.46}N_{0.19} + CO_2 + H_2O + H^+ \quad (1)$$

Under stress conditions such as under O_2 and/or NH_4^+-N limitation, *C. necator* has the capability of producing pure PHB (homopolymer) using an organic carbon (Eq. (2)) or using CO_2 as carbon source and H_2 as energy source (Eq. (3)) [12].

$$C_xH_yO_z + O_2 \rightarrow C_4H_6O_2 + CO_2 + H_2O \quad (2)$$

$$33H_2 + 12O_2 + 4CO_2 \rightarrow C_4H_6O_2 + 30H_2O \quad (3)$$

Although the heterotrophic-autotrophic process has a high potential to produce PHB from CO_2, it only attracted a limited amount of research attention so far, only focusing on experimental work. Tanaka and Ishizaki [10] proved the feasibility of the concept using fructose as the carbon source during the heterotrophic growth phase and a gas mixture of CO_2:H_2:O_2 = 10.3:84.1:6.7 during the autotrophic PHB production phase. Sugimoto and his collaborators [7] used acetic acid for heterotrophic growth but found a low PHB productivity in the subsequent phase due to the inhibitory effect of acetic acid on enzymes related to autotrophic metabolism. Recently the feasibility of heterotrophic-autotrophic PHB production was demonstrated using either glucose or waste glycerol for the heterotrophic growth phase and maintaining an O_2 fraction lower than 3% in the gas mixture during the autotrophic phase, to ensure safe operational conditions [12].

The microbial production of PHB comprises a number of process steps including cell growth, intracellular PHB accumulation, PHB consumption etc. in which a lot of process variables and other influencing factors are involved [13]. This makes optimal process design and operation for efficient production of PHB one of the main challenges. Modelling and simulation are very useful tools to understand the dynamic process behaviour, the underlying mechanisms and to develop control strategies for maximizing PHB production [14]. However, up till now no model is available for heterotrophic-autotrophic PHB production.

This study constitutes the first contribution in which modelling and simulation was applied for optimizing heterotrophic-autotrophic PHB production. The process was described combining previously established models for heterotrophic–heterotrophic [15] and autotrophic–autotrophic [16] PHB production processes. The model was validated on the experimental datasets of Garcia-Gonzalez et al. [12]. Subsequently, the model was applied in view of process optimization in terms of maximizing PHB production, to examine the influence of the O_2 and NH_4^+-N concentration in the fermentation medium.

2. Modeling heterotrophic-autotrophic PHB production

2.1. Process stoichiometry and kinetics

The model for heterotrophic-autotrophic PHB production was based on previously established models for heterotrophic–heterotrophic PHB production [15] and for autotrophic–autotrophic PHB production [16], for the same microorganism as in this study, *Cupriavidus necator* DSM 545. The detailed model stoichiometry and kinetics are given in Table 1 and Table 2, respectively and all the parameter values in Table S1 in the Supplementary materials.

Four processes were considered: heterotrophic biomass growth on organic substrate and on PHB, heterotrophic PHB production on organic substrate and autotrophic PHB production using CO_2 as carbon source. Maintenance on organic substrate was not taken into account because it does not have significant effect during the heterotrophic growth phase [15,17]. Heterotrophic cell growth (phase

Table 2
Process kinetics for heterotrophic-autotrophic PHB production.

Process	Kinetic expression
1. Biomass growth on substrate	$\mu_{xs=}\mu_{xs}^{max}\left(\frac{S}{K_s+S+\frac{S^2}{K_{IS}}}\right)\left(\frac{N}{K_N+N+\frac{N^2}{K_{IN}}}\right)\left(\frac{O_2}{K_{xO2}+O_2}\right)\left[1-\left(\frac{X}{X_m}\right)^{\alpha}\right]$
2. Biomass growth on PHB	$\mu_{xp=}\mu_{xp}^{max}\frac{f_{PHB}}{K_{PHB}+f_{PHB}}\left(\frac{N}{K_N+N+\frac{N^2}{K_{IN}}}\right)\left(\frac{O_2}{K_{xO2}+O_2}\right)\left[1-\left(\frac{X}{X_m}\right)^{\alpha}\right]$
3. PHB production on organic substrate	$\mu_{ps_{het}=}\mu_{ps_{het}}^{max}\left(\frac{S}{K_{ps}+S+\frac{S^2}{K_{PIS}}}\right)\left(\frac{O_2}{K_{pO2}+O_2+\frac{O_2^2}{K_{PIO2}}}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB(max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N+K_{PIN}}$
4. PHB production on gaseous substrate	$\mu_{ps_{aut}}=\mu_{ps_{aut}}^{max}\left(\frac{H_2}{K_{pH2}+H_2}\right)\left(\frac{O_2}{K_{pO2}+O_2+\frac{O_2^2}{K_{PIO2}}}\right)\left(\frac{CO_2}{K_{pCO2}+CO_2}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB(max)}}\right)^{\beta}\right]\frac{K_{PIN}}{N+K_{PIN}}$

1), both on organic substrate and on PHB, is limited by too low concentrations of NH_4^+-N and inhibited when these concentrations are too high, which is modelled through Haldane kinetics. The effect of organic substrate on cell growth is also described by Haldane kinetics, while growth on PHB is limited (not inhibited) by the intracellular PHB fraction. Monod kinetics were included to describe the limitation effect of O_2 on biomass growth; it was assumed that the O_2 concentration never reached inhibitory levels. Cell density inhibition of biomass growth on substrate and on PHB were taken into account by a modified logistic growth expression [15,17]. During the heterotrophic growth phase (phase 1), the headspace gas was at atmospheric pressure and its composition was similar to the one of air. CO_2 produced during the heterotrophic growth phase was not considered in the model because its concentration was very low and it was not used as a carbon source due to the absence of hydrogen (H_2) in this phase. The limitation and inhibition effect of oxygen on heterotrophic PHB production [18] was modelled through Haldane kinetics. A fixed oxygen (O_2) concentration was assumed during phase 1; during the PHB production phase (phase 2) O_2 was considered as a state variable. The effect of O_2 on autotrophic PHB production (phase 2) was modelled through Haldane kinetics [16]. Due to the low solubility, the concentrations of H_2 during autotrophic PHB production was reasonably assumed not to be in the inhibiting range and the limitation effect was modelled through Monod kinetics. CO_2 causes significant cell damage and thus reduces PHB production at headspace concentrations of 22% [19]. However, in this study the CO_2 composition in gas phase was maintained below 15%, which is in the limiting range. Monod kinetics were used to describe the CO_2 limitation effect. The stoichiometric coefficients were determined by the stoichiometric equation for autotrophic PHB production (Eq. (3)), which was based on the *overall* consumption of gaseous substrate and on the *overall* PHB production [6]. In this way, maintenance was lumped in the stoichiometry and therefore not considered as a separate process. Autotrophic biomass growth was not considered as the autotrophic phase was only started when the NH_4^+-N concentration had become very low, thus preventing biomass growth.

Distinct state variables were defined to describe PHB produced from organic substrate (P_{het}) or from CO_2 (P_{aut}). This model feature can be seen as a 'labelling' of produced PHB to track its origin. It is clear that no physical distinction exists between PHB produced in a heterotrophic or autotrophic way. The fraction of PHB into the cell is defined as PHB to residual cell ratio (f_{PHB}). Both heterotrophic and autotrophic PHB production are inhibited by NH_4^+-N, which was expressed by a non-competitive inhibition equation; product (PHB) inhibition was modelled through a modified logistic expression (Table 2).

2.2. Mass balances

The mass balances over the fermentor for heterotrophic-autotrophic PHB production comprise advective transport (inflows and outflows, interphase transport (between the gas and the liquid phase)) and biochemical conversion (transformation of substrate and nutrients into PHB or residual biomass). The corresponding terms for the heterotrophic growth phase and for the autotrophic PHB production phase were described in Mozumder et al. [15] and in Mozumder et al. [16] respectively.

The feed flow rates of the organic substrate and nutrient solutions during the heterotrophic growth phase were determined from the mass balances for organic substrate and nitrogen, given that their concentrations were maintained at a constant (optimal) level.

$$\frac{dS(t)}{dt} = \frac{F_S(t)S_F}{V(t)} - D(t)S - \mu_S X(t) = 0 \tag{4}$$

$$\frac{dN(t)}{dt} = \frac{F_N(t)N_F}{V(t)} - D(t)N - \mu_N X(t) = 0 \tag{5}$$

μ_S (g substrate/g cell/h) and μ_N (g nitrogen/g cell/h) denote the specific substrate consumption rate and the specific nitrogen consumption rate, respectively.

$$\mu_S = \frac{\mu_{xs}}{Y_{xs}} + \frac{\mu_{ps_{het}}}{Y_{ps}} \tag{6}$$

$$\mu_N = \frac{\mu_{xs} + \mu_{xp}}{Y_{xN}} \tag{7}$$

Assuming that neither biomass nor PHB were present in the feed solutions, the biomass and PHB concentration profiles during heterotrophic phase were obtained from their respective mass balances.

$$\frac{dX(t)}{dt} = (\mu_x - D(t))X(t) \tag{8}$$

$$\frac{dP_{het}(t)}{dt} = \mu_{ps_{het}}X - D(t)P_{het}(t) \tag{9}$$

in which μ_x (g cell/g cell/h) and $\mu_{p_{het}}$ (g PHB/g cell/h) denote the net biomass growth rate and the net PHB production rate, respectively.

$$\mu_x = \mu_{xs} + \mu_{xp} \tag{10}$$

$$\mu_{p_{het}} = \mu_{ps_{het}} - \frac{\mu_{xp}}{Y_{xp}} \tag{11}$$

During autotrophic PHB production, the reactor volume remains constant since there is no liquid feeding to the system. The macroscopic transport comprises mass transfer from the gas phase to the liquid phase. The individual mass balances are represented by Eqs. (12)–(14).

$$\frac{dH_2(t)}{dt} = k_L a_{(H_2)}\left(H_2^* - H_2\right) - \left(\frac{\mu_{ps_{aut}}}{Y_{pH2}}\right)X \tag{12}$$

$$\frac{dO_2(t)}{dt} = k_L a_{(O_2)}\left(O_2^* - O_2\right) - \left(\frac{\mu_{ps_{aut}}}{Y_{pO2}}\right)X \tag{13}$$

$$\frac{dCO_2(t)}{dt} = k_L a_{(CO_2)}\left(CO_2^* - CO_2\right) - \left(\frac{\mu_{ps_{aut}}}{Y_{pCO2}}\right)X \tag{14}$$

H_2^*, O_2^*, CO_2^* denote the equilibrium liquid phase concentrations corresponding with the gas phase composition of H_2, O_2, CO_2 respectively as expressed by Henry's law (Eq. (15)). The solubility of a gas ($C^*\left(H_2^*, O_2^*, CO_2^*\right)$, g/L) is the inverse of Henry's constant (k_H, atm/g/L), multiplied by the partial pressure of the gas (P_g, atm).

$$C^* = P_g/k_H \tag{15}$$

The Henry's constant (k_H) of each gas was calculated from the gas solubility at standard conditions (pure gases at 30 °C and 1 atm pressure [20], see Table S1 in Supplementary materials).

The overall volumetric mass transfer coefficients for H_2 ($k_L a_{H2}$) and for CO_2 ($k_L a_{CO2}$) were calculated from that of O_2 ($k_L a_{O2}$) according to Eq. (16) [21] and Eq. (17) [22] respectively.

$$k_L a_{H2} = 0.280(k_L a_{O2})^{1.29} \tag{16}$$

$$k_L a_{CO2} = \sqrt{\frac{D_{lCO2}}{D_{lO2}}}k_L a_{O2} \tag{17}$$

In which D_{lCO2} is the diffusion coefficient for CO_2 (1.77×10^{-5} cm^2/s [23]) and D_{lO2} is the diffusion coefficient for O_2 (2.50×10^{-5} cm^2/s [22]).

Table 3
Dependency of maximum PHB to RCC ratio, $f_{PHB(max)}$ on organic substrate type and autotrophic phase conditions.

Organic substrate in heterotrophic phase	RCC at the autotrophic phase	$f_{PHB(max)}$	Reference
Fructose	4	5.6	[10]
Fructose	10	1.3	[10]
Fructose	15	1.3	[10]
Acetic acid	5	1.2	[7]
Glucose	5	(16/5) = 3.2	[12]
Glucose	16	(11/16) = 0.7	[12]
Waste glycerol	5	(13/5) = 2.6	[12]
Waste glycerol	16	(28/16) = 1.8	[12]

The autotrophic PHB production rate depends on the specific PHB production rate ($\mu_{ps_{aut}}$) and residual cell concentration (RCC) in the autotrophic phase, as expressed by the PHB mass balance:

$$\frac{dP_{aut}(t)}{dt} = \mu_{ps_{aut}} X \quad (18)$$

The total PHB production in the heterotrophic-autotrophic system was calculated as the sum of the amount of PHB produced during the heterotrophic growth condition (P_{het}) (Eq. (9))and the produced PHB under autotrophic condition (P_{aut}) (Eq. (18)).

$$\frac{dP(t)}{dt} = \frac{dP_{het}(t)}{dt} + \frac{dP_{aut}(t)}{dt} = (\mu_{ps_{aut}} + \mu_{ps_{het}})X - D(t)P_{het}(t) \quad (19)$$

2.3. Model validation

The model was applied to simulate the four experimental datasets described by Garcia-Gonzalez et al. [12]. The model fit was evaluated through visual comparison and by applying the Nash-Sutcliffe model efficiency coefficient (E) [24] as a quantitative means to assess the accuracy of model outputs and thus the predictive power of the model. Essentially, the closer the model efficiency is to 1, the more accurate the model is.

2.4. Scenario analysis

The validated model was applied for scenario analysis, to determine the optimal operation conditions, in terms of O_2 and NH_4^+-N concentrations in the fermentation medium maximizing the specific biomass growth rate and the specific PHB production rate. In all cases glucose was used as carbon source during heterotrophic growth (phase 1) and NH_4^+-N limitation was imposed at 5 g/L residual cell concentration (RCC) to shift to the PHB production phase ($f_{PHB(max)}$ = 3.2). A gas mixture of H_2, O_2 and CO_2 was supplied for autotrophic PHB production (phase 2).

3. Results and discussion

3.1. Model validation

The model for heterotrophic-autotrophic PHB production was validated on the experimental datasets reported by Garcia-Gonzalez et al. [12]. These datasets differed in the substrates applied during the heterotrophic growth phase, being pure glucose or waste glycerol, and in the residual cell concentration (RCC) for which the autotrophic PHB production phase was initiated, namely around 5 and 16 g/L RCC. Fig. S1 (in Supplementary materials) displays the model validation results for heterotrophic-autotrophic PHB production using glucose or waste glycerol for growth. During the heterotrophic growth phase, sufficient substrate (initially 10 g/L glucose or 12 g waste glycerol), O_2 (55% of air saturation) and NH_4^+-N (initially 0.75 g/L NH_4^+-N) concentrations were maintained to favour growth instead of PHB production. Approximately 21 h and 22 h were needed to reach 5 g/L RCC using glucose and waste glycerol as a substrate, respectively; while to reach 16 g/L RCC the duration was around 27 h and 29 h, respectively. When the desired RCC was achieved, NH_4^+-N limitation was imposed to stimulate PHB production, by stopping the NH_4^+-N feeding. The organic substrate feeding was stopped and a gas mixture of H_2:O_2:CO_2 = 84:2.8:13.2 vol% was supplied, thus realizing autotrophic conditions.

The model described the experimental observations obtained with glucose quite well, with Nash-Sutcliffe model efficiency coefficients E = 0.91 and 0.92 for RCC and E = 0.87 and 0.81 for PHB when the autotrophic phase started at 5 and 16 g/L RCC respectively (Fig. S1a–b). Also for the experimental data using waste glycerol for growth, the model predictions agreed well with the experimental results in terms of RCC, PHB and total biomass concentration (Fig. S1c–d). The E-values corresponding with an operation of autotrophic phase at 5 and 16 g/L RCC are 0.95 and 0.82 for RCC and 0.86 and 0.97 for PHB, all of which are close to 1, thus indicating a good model fit. The final biomass and PHB concentrations were however not so well predicted (Fig. S1 in Supplementary materials) by taking a fixed value of $f_{PHB(max)}$. In order to fit the final PHB concentration, it was found required to adjust the $f_{PHB(max)}$. By adjusting the value of the maximum PHB to RCC ratio, $f_{PHB(max)}$, according to the experimental data obtained by Garcia-Gonzalez et al. [12] (Table 3), instead of using the default value of $f_{PHB(max)}$(=1.78), the final biomass and PHB concentration was very well predicted (Fig. 1). Moreover, by adjusting $f_{PHB(max)}$ value the overall PHB productivity (g/L/h) was in better agreement with the experimental observation (Table S2 in Supplementary materials).

Apparently the maximum PHB to RCC ratio obtained during autotrophic PHB production, $f_{PHB(max)}$, depends on the type of organic substrate and on the duration of heterotrophic growth phase. The ratio $f_{PHB(max)}$ decreased when delaying the start of the autotrophic PHB production phase (Table 3). This confirms that the specific activity of the enzymes linked to autotrophic metabolism but synthetized during heterotrophic growth were considerably affected by organic substrate and a prolonged heterotrophic growth phase, as indicated by Friedrich et al. [25]. Further research is recommended to elucidate the mechanisms behind both phenomena, leading to the establishment of correlations for their description, which could be integrated into the model.

During the heterotrophic growth phase, organic substrate and NH_4^+-N were fed in stoichiometric amounts such that they were both consumed when switching to the autotrophic PHB production phase. While an ideal stoichiometric ratio can easily be applied and maintained during simulation, in practice dosing of NH_4^+-N or glucose had to be adjusted just before switching to the second phase, due to ammonia stripping and/or inaccuracies in the feeding equipment. Besides, transition between the two phases took around 30–40 min during which sometimes a small amount of (organic) substrate had to be provided to guarantee the survival of the bacteria. Nevertheless, the simulation results matched the experimental data very well.

The good agreement between the experimental data and simulation results is remarkable, given that no model calibration was performed for heterotrophic-autotrophic PHB production as such,

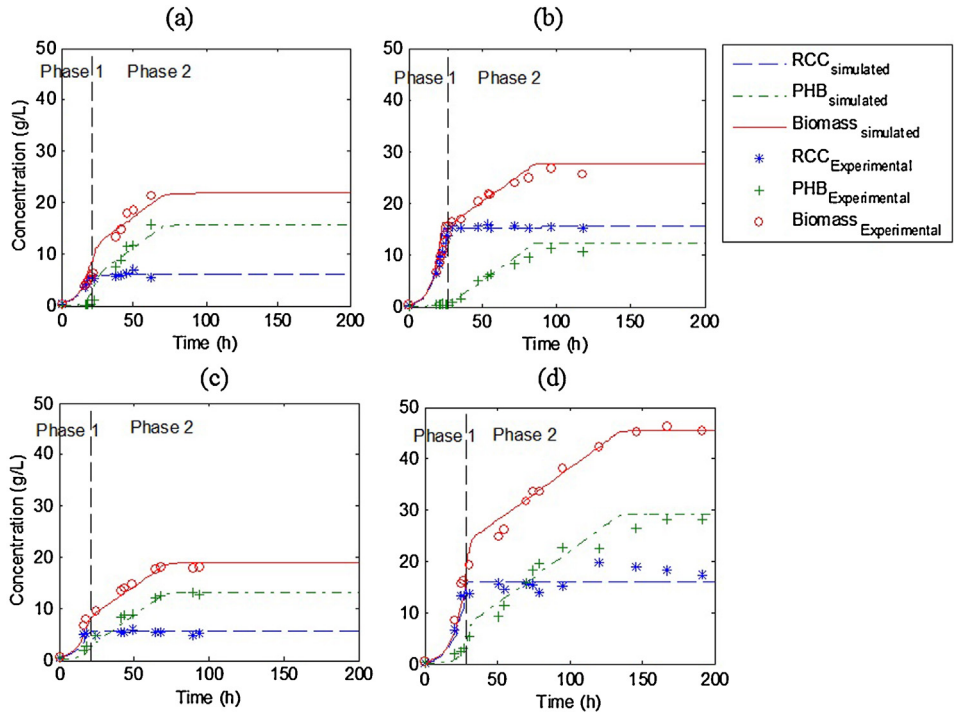

Fig 1. Validation of the model for heterotrophic-autotrophic PHB production, with adjustment of $f_{PHB(max)}$ according to Table 3. Comparison between the simulation outcome (full lines) and experimental observations (discrete markers) using glucose (a, b) and waste glycerol (c, d) as a substrate in the heterotrophic phase (phase 1) and starting the autotrophic phase at 5 g/L RCC (a, c) and 16 g/L RCC (b, d).

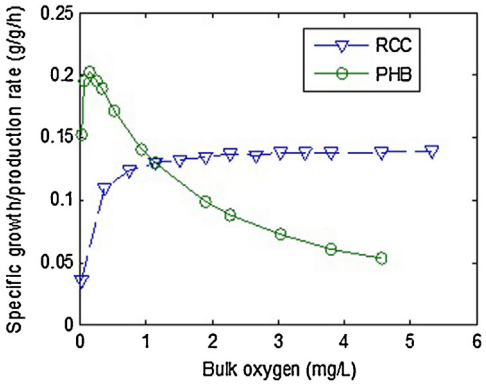

Fig. 2. Effect of O_2 concentration on specific biomass (RCC) growth rate and specific PHB production rate. Biomass growth was conducted at 0.70 g/L NH_4^+-N; PHB production was under NH_4^+-N –free (NH_4^+-N = 0 g/L) conditions.

but the model structure and all model parameters – except $f_{PHB(max)}$ – were obtained for heterotrophic–heterotrophic PHB production [15] and autotrophic–autotrophic PHB production [16]. This underlines the general applicability of the developed model.

3.2. Analysis of the heterotrophic-autotrophic PHB production process

3.2.1. Effect of dissolved oxygen concentration on growth and PHB production

The role of the dissolved oxygen (DO) concentration on specific biomass growth and specific PHB production rate was examined through model simulation. During the growth phase (phase 1) NH_4^+-N was maintained at 0.7 g/L, while the PHB production phase (phase 2) was NH_4^+-N free (NH_4^+-N = 0 g/L). Fig. 2 displays the total specific biomass growth rate, on substrate and on PHB, and the total specific PHB production rate, both autotrophic and heterotrophic, in terms of the DO concentration. The biomass growth rate remained almost the same for DO concentrations above 1.5 mg/L (equivalent to 20% air saturation concentration) – note that the model implicitly assumed that the O_2 concentration never reached inhibitory levels for growth. Below 1.5 mg O_2/L, the growth rate decreased due to O_2 limitation.

As for PHB production, the optimal oxygen concentration was found at 0.224 mg/L (equivalent to 3% air saturation concentration). This value corresponds to the maximum of the Haldane inhibition kinetics through which the effect of oxygen is described. Below and above this concentration the specific PHB production rate decreases due to limitation and inhibition, respectively. The sharp peak of specific PHB production reflects that the O_2 half saturation and inhibition constant are very close to each other, so the maximum PHB production rate is very sensitive to the O_2 concentration.

The optimal O_2 concentration for maximum biomass growth is higher than the one for maximum PHB production. This is not only true for heterotrophic biomass growth, but also for autotrophic biomass growth. During autotrophic growth on CO_2 and H_2, the high optimal O_2 levels to reach maximum biomass growth cannot be maintained because of process safety (explosion risk). For this reason, the heterotrophic-autotrophic process is preferred over the autotrophic–autotrophic PHB production process [10].

3.2.2. Effect of ammonium-nitrogen (NH_4^+-N) on growth and PHB production

The effect of NH_4^+-N concentration on biomass growth and PHB production is summarized in Fig. 3. The O_2 concentration was maintained at 4.18 mg/L in growth phase (55% air saturation, as in [26]) and at 2.28 mg/L (30% air saturation) in PHB production phase. The simultaneous effect of both oxygen and ammonium-nitrogen concentration on specific biomass growth rate over substrate (μ_{xs}) and specific PHB production rate on gaseous substrate ($\mu_{ps_{aut}}$) is summarized in Fig. S2 and S3 (in Supplementary materials) respectively.

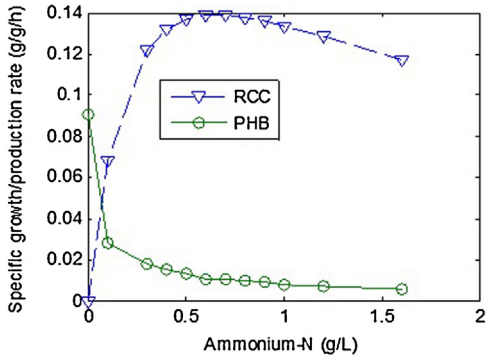

Fig. 3. Effect of NH_4^+-*N* concentration on total specific biomass (RCC) growth rate and total specific PHB production rate. Biomass growth was conducted at 4.18 mg/L and PHB production was at 2.28 mg/L O_2 concentration.

NH_4^+-*N* has both a limitation and inhibition effect on growth; the maximum specific growth rate was observed at NH_4^+-*N* concentration of 0.6–0.7 g/L. Below this concentration the growth rate decreased due to limitation; a linear decrease of the maximum specific growth rate with increasing NH_4^+-*N* concentrations above this value was observed due to inhibition. This linear decrease corresponds with the findings of Belfares et al. [27] for the same organism (*C. necator*) who reported a linear inhibition effect at NH_4^+-*N* concentration above 2 g/L. Gahlawat and Srivastava [28] found the same optimal NH_4^+-*N* concentration of 0.7 g/L for Azohydromonas australica, of which the growth rate increased with increasing NH_4^+-*N* concentrations below this value and then decreased, first slowly and then rapidly once beyond 11 g/L NH_4^+-*N*. It should be noted however that there are no benefits in operating at such high NH_4^+-*N* concentrations. In most fed-batch experiments, NH_4^+-*N* feeding is regulated by the pH control, using NH_4OH as a base. The ratio of NH_4^+-*N* consumption and H^+ production during the growth (phase 1) is 1:1 (according to Eq. (1)), therefore there is no possibility of overfeeding or accumulation of NH_4^+-*N*, such that NH_4^+-*N* inhibition will not occur in practice. However, during growth the fermentation medium is rich in NH_4^+-*N*, magnesium and phosphate, which could lead to the production of struvite ($NH_4MgPO_4{\cdot}6H_2O$) crystals [29], resulting in a decreased NH_4^+-*N* concentration, as observed by Mozumder et al. [26] during the growth phase. So from a technical point of view, the most challenging part in realizing the optimal NH_4^+-*N* concentration corresponding with the maximum growth rate, is to overcome the NH_4^+-*N* limitation effect. The specific PHB production rate was the highest when NH_4^+-*N* became zero (Fig. 3), which is in agreement with the fact that NH_4^+-*N* stress is applied to stimulate PHB production [30,31].

4. Conclusions

- Heterotrophic-autotrophic PHB production was described based on existing models for heterotrophic–heterotrophic PHB production and autotrophic–autotrophic PHB production, and applying parameter values previously obtained for these cases. The simulation results matched experimental data very well, without the need for additional model calibration except for the maximum PHB to residual cell ratio.
- Both the type of organic substrate used in growth phase and the duration of heterotrophic growth phase negatively affect the autotrophic PHB production capacity (maximum PHB to residual cell ratio) in the heterotrophic-autotrophic PHB production process. Further research is recommended to establish correlations in this respect.
- While it is generally known that oxygen stress conditions stimulate PHB production, too low O_2 concentrations may have an adverse effect because of oxygen limitation. The optimal O_2 concentration for PHB production was determined as 0.224 mg/L. Above this value, PHB production was restricted due to inhibition.
- The optimal NH_4^+-*N* concentration for biomass growth was 0.60-0.70 g/L NH_4^+-*N*, while PHB production was maximal under NH_4^+-*N* free condition.

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.bej.2016.07.007.

References

[1] S. Chanprateep, Current trends in biodegradable polyhydroxyalkanoates, J. Biosci. Bioeng. 110 (2010) 621–632.
[2] S. Obruca, P. Benesova, L. Marsalek, I. Marova, Use of lignocellulosic materials for PHA production, Chem. Biochem. Eng. Q. 29 (2015) 135–144.
[3] M. Koller, G. Braunegg, Biomediated production of structurally diverse poly(hydroxyalkanoates) from surplus streams of the animal processing industry, Polimery 60 (2015) 298–308.
[4] J. Pais, L.S. Serafim, F. Freitas, M.A.M. Reis, Conversion of cheese whey into poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by *Haloferax mediterranei*, New Biotechnol. 33 (2016) 224–230.
[5] C. Hermann-Krauss, M. Koller, A. Muhr, H. Fasl, F. Stelzer, G. Braunegg, Archaeal production of polyhydroxyalkanoate (PHA) co-and terpolyesters from biodiesel industry-derived by-products, Archaea (2013) 1–10.
[6] A. Ishizaki, K. Tanaka, Production of poly-β-hydroxybutyric acid from carbon dioxide by *Alcaligenes eutrophus* ATCC 17697T, J. Ferment. Bioeng. 71 (1991) 254–257.
[7] T. Sugimoto, T. Tsuge, K. Tanaka, A. Ishizaki, Control of acetic acid concentration by pH-stat continuous substrate feeding in heterotrophic culture phase of two-stage cultivation of *Alcaligenes eutrophus* for production of P(3HB) from CO_2 H_2, and O_2 under non-explosive conditions, Biotechnol. Bioeng. 62 (1999) 625–631.
[8] T.G. Volova, E.G. Kiselev, E.I. Shishatskaya, N.O. Zhila, A.N. Boyandin, D. Syrvacheva, O.N. Vinogradova, G.S. Kalacheva, A.D. Vasiliev, I.V. Peterson, Cell growth and accumulation of polyhydroxyalkanoates from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646, Bioresour. Technol. 146 (2013) 215–222.
[9] T. Takeshita, A. Ishizaki, Influence of hydrogen limitation on gaseous substrate utilization in autotrophic culture of *Alcaligenes eutrophus* ATCC 17697T, J. Ferment. Bioeng. 81 (1996) 83–86.
[10] K. Tanaka, A. Ishizaki, Production of poly-D-3-hydroxybutyric acid from carbon dioxide by a two-stage culture method employing *Alcaligenes eutrophus* ATCC 17697T, J. Ferment. Bioeng. 77 (1994) 425–427.
[11] H.G. Schlegel, G. Gottschalk, R. Von Bartha, Formation and utilization of poly-beta-hydroxybutyric acid by Knallgas bacteria (Hydrogenomonas), Nature 191 (1961) 463–465.
[12] L. Garcia-Gonzalez, M.S.I. Mozumder, M. Dubreuil, E.I.P. Volcke, H. De Wever, Sustainable autotrophic production of polyhydroxybutyrate (PHB) from CO_2 using a two-stage cultivation system, Catal. Today 257 (2015) 237–245.
[13] G. Penloglou, C. Chatzidoukas, C. Kiparissides, Microbial production of polyhydroxybutyrate with tailor-made properties: an integrated modelling approach and experimental validation, Biotechnol. Adv. 30 (2012) 329–337.
[14] M. Novak, M. Koller, G. Braunegg, P. Horvata, Mathematical modelling as a tool for optimized PHA production, Chem. Biochem. Eng. Q. 29 (2015) 183–220.
[15] M.S.I. Mozumder, L. Goormachtigh, L. Garcia-Gonzalez, H. De Wever, E.I.P. Volcke, Modeling pure culture heterotrophic production of polyhydroxybutyrate (PHB), Bioresour. Technol. 155 (2014) 272–280.
[16] M.S.I. Mozumder, L. Garcia-Gonzalez, H. De Wever, E.I.P. Volcke, Poly(3-hydroxybutyrate) (PHB) production from CO_2: model development and process optimization, Biochem. Eng. J. 98 (2015) 107–116.
[17] M.S.I. Mozumder, L. Garcia-Gonzalez, H. De Wever, E.I.P. Volcke, Effect of sodium accumulation on heterotrophic growth and polyhydroxybutyrate (PHB) production by *Cupriavidus necator*, Bioresour. Technol. 191 (2015) 213–218.
[18] C. Chatzidoukas, G. Penloglou, C. Kiparissides, Development of a structured dynamic model for the production of polyhydroxybutyrate (PHB) in *Azohydromonas lata* cultures, Biochem. Eng. J. 71 (2013) 72–80.
[19] L. Shang, M. Jiang, C. Hee Ryu, H.N. Chang, S.H. Cho, J.W. Lee, Inhibitory mEffect of carbon dioxide on the fed-Batch culture of *Ralstonia eutropha*: evaluation by CO_2 pulse injection and autogenous CO_2 methods, Biotechnol. Bioeng. 83 (2003) 312–320.
[20] J.A. Dean, Lange's Handbook of Chemistry, thirteenth ed., McGraw-Hill Book Company, New York, 1985.

[21] A. Ishizaki, K. Tanaka, N. Taga, Microbial production of poly-D-3-hydroxybutyrate from CO_2, Appl. Microbiol. Biotechnol. 57 (2001) 6–12.
[22] B. de Heyder, P. Vanrolleghem, H. Van Langenhove, W. Verstraete, Kinetic characterization of mass transfer limited biodegradation of a low water soluble gas in batch experiments – necessity for multiresponse fitting, Biotechnol. Bioeng. 55 (1997) 511–519.
[23] R.E. Treybal, Mass Transfer Operation, MaGraw-Hill Book Company, New York, 1955.
[24] J.E. Nash, J.V. Sutcliffe, River flow forecasting through conceptual models part I – a discussion of principles, J. Hydrol. 10 (1970) 282–290.
[25] C. Friedrich, B. Friedrich, B. Bowien, Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*, J. Gen. Microbiol. 16 (1981) 69–78.
[26] M.S.I. Mozumder, H. De Wever, E.I.P. Volcke, L. Garcia-Gonzalez, A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production, Process Biochem. 49 (2014) 365–373.
[27] L. Belfares, M. Perrier, B.A. Ramsay, J.A. Ramsay, M. Jolicoeur, C. Chavarie, Multi-inhibition kinetic model for the growth of *Alcaligenes eutrophus*, Can. J. Microbiol. 41 (1995) 249–256.
[28] G. Gahlawat, A.K. Srivastava, Development of amathematical model for the growth associated Polyhydroxybutyrate fermentation by *Azohydromonas australica* and its use for the design offed-batch cultivation strategies, Bioresour. Technol. 137 (2013) 98–105.
[29] S.A. Scudder, The precipitation of magnesium ammonium phosphate crystals during the growth of bacteria in media containing nitrogenous substances, J. Bacteriol 16 (1928) 157–161.
[30] J.M.B.T. Cavalheiro, R.S. Raposo, C.M.D. de Almeida, M.T. Cesario, C. Sevrin, C. Grandfils, M.M.R. da Fonseca, Effect of cultivation parameters on the production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by *Cupriavidus necator* using waste glycerol, Bioresour. Technol. 111 (2012) 391–397.
[31] D. Tan, Y.S. Xue, G. Aibaidula, G.Q. Chen, Unsterile and continuous production of polyhydroxybutyrate by *Halomonas* TD01, Bioresour. Technol. 102 (2011) 8130–8136.