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Simultaneous growth and poly(3-hydroxybutyrate) (PHB) accumulation in a *Plasticicumulans acidivorans* dominated enrichment culture

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ABSTRACT

The wide variety of organic carbon to nitrogen and phosphorous ratios that are encountered in different wastewaters has a major impact on the poly(3-hydroxybutyrate) (PHB) accumulation potential of microbial communities. In this study we investigated the influence of the substrate composition in terms of the carbon to nitrogen (C/N) or phosphorus (C/P) ratio on the PHB accumulation performance. A multi-reactor set-up was used, enabling parallel experiments using identical inoculum of an enrichment culture dominated by *Plasticicumulans acidivorans*. In all experiments simultaneous PHB production and growth was observed. Generally, when trace amounts of growth nutrients were present the PHB production yield on substrate remained high for at least 12 h. Interestingly, from the carbon to nutrient ratio in the substrate, the PHB wt% could be accurately predicted in the accumulations. This study demonstrates that strict uncoupling of microbial growth and PHA accumulation is not required for achieving high cellular PHA-contents. Herewith the range of wastewaters that enable a cellular PHA content of 80 % or higher for at least 12 h is expanded to C:N and C:P-ratios exceeding COD:N of 26 gCOD:gNH_4-N and COD:P of 511 gCOD:gPO_4-P respectively.

1. Introduction

Many products used on a daily basis are fossil-fuel derived. These resources are finite, making alternative raw materials desired. A renewable source that contains large amounts of organic matter is (industrial) wastewater. In the last decade(s) the recovery of various products from wastewater has gotten significant attention. Products vary from biogas -an established technology- to innovative products such as extracellular polymeric substances (EPS) from granular sludge and bioplastics production (Kleerebezem et al., 2015; Van Der Hoek et al., 2016).

One way of producing bioplastics from (industrial) wastewater is using enrichment cultures. Poly(3-hydroxybutyrate) (PHB) is a type of bioplastic produced by microorganisms in which it can serve as microbial storage polymer. One effective enrichment technique for obtaining PHB producing microorganisms is pulse feeding the culture, creating periods where there is a surplus of organic substrate and times of starvation. Microorganisms that consume the substrate the fastest store this as PHB and thus can proliferate during the starvation periods in which substrate is absent. This so-called feast-famine regime creates a situation in which the bioplastic producing microbes will dominate the enrichment. The PHB content of the enriched microbes is maximized in an accumulation, a (fed)-batch process in which preferably no essential growth nutrient such as ammonium or phosphate are present. Using this technology, cultures have been enriched which can store up to 9 times their weight as bioplastic (0.90 gPHA·$gVSS^{-1}$) (Jiang et al., 2011a; Marang et al., 2013).

Lab and pilot studies have demonstrated that the feasibility of waste-based bioplastic production strongly depends on the composition of the waste stream in terms of carbon source and presence of nutrients (Johnson et al., 2010a, 2010b; Korkakaki et al., 2017, 2016a; Valentino et al., 2018). In general lower polyhydroxyalkanoates (PHA) contents are achieved at pilot scale compared to lab scale studies. Solids present in the influent lower the final PHA purity. Furthermore, waste streams can contain varying amounts of growth nutrients such as ammonium and phosphate. These growth nutrients could be disadvantageous for the

Abbreviations: PHB, poly(3-hydroxybutyrate); PHA, polyhydroxyalkanoates; VFA, volatile fatty acids; OFMSW, organic fraction of municipal solid waste; HRT, hydraulic retention time; N-limited, nitrogen limited; P-limited, phosphorus limited; TSS, total suspended solids; VSS, volatile suspended solids; EPS, extra polymeric substances.

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PHB production process as microbial growth cannot be excluded to occur in the accumulation phase. Previous studies have shown the disadvantageous effect of growth nutrients present in the PHA accumulation process as generally lower overall PHA purities were obtained (Johnson et al., 2010a; Morgan-Sagastume et al., 2015). There are techniques present for pre-removal of ammonium and phosphorus. Phosphorus and nitrogen can be removed through biological or physical chemical treatment to reach growth-limited streams (Huang et al., 2018; Ndam et al., 2018). However, the cost-effective removal of growth nutrients remains in many cases a challenge.

The growth nutrients present in a waste stream can also be used as an advantage rather than being considered a disadvantage. The growth nutrients can potentially be used to have growth and PHA production simultaneously. The aim of this study was to investigate the overall PHB producing potential at different C/N or C/P ratios using a pre-enriched PHA producing specialist (*Plasticicumulans acidivorans*) as inoculum (Jiang et al., 2011b). Accumulation experiments were conducted in parallel using an inoculum obtained from the same steady-state enrichment reactor. Each accumulation reactor was fed with a designed synthetic medium that contained excess carbon and either ammonium or phosphate as limiting growth nutrient. This study aimed to elucidate in more detail the requirement of nutrient limitation for the production of PHB. Additionally, this study may provide insight in the question if cultivation and accumulation need to be uncoupled, or that some degree of growth during accumulation can benefit the overall PHA process.

2. Materials and methods

2.1. Inoculum cultivation

The inoculum for each fed-batch experiment was the effluent from a PHB producing enrichment dominated for at least 90 % by *Plasticicumulans acidivorans*. This enrichment was maintained in a bioreactor, operated at 30 ± 1 °C using a warm water jacket on the outside of the reactor. The bioreactor had a working volume of 1.4 L. The pH of the reactor broth was measured and controlled at 7.0 ± 0.1 using 1 M of HCl or 1 M NaOH. To ensure no oxygen and mixing limitation the liquid broth was aerated at a rate of 200 mL min^{-1} using a fine bubble dispenser and was mixed by a stirrer at 750 rpm. The reactor was operated as a sequencing batch reactor (SBR) according to settings from a previous study (Johnson et al., 2009). A hydraulic retention time (HRT) of 24 h was applied with a cycle length of 12 h. The only difference with Johnson et al. (2009) was an additional amount of trace elements dosed to the bioreactor, we used a final concentration 3.75 $mL{\cdot}L^{-1}$ of Vishniac trace element solution (Vishniac and Santer, 1957). The presence of *P. acidivorans* in the enrichment was verified and estimated by Fluorescent In Situ Hybridization (FISH) microscopy. In the enrichment reactor the amount of phosphorus or nitrogen supplied was lowered in the cycle prior to the accumulation, minimizing residual phosphorus or nitrogen concentrations

2.2. Fed-batch set-up

The fed-batch experiments were performed in 4 separate identical bioreactors, identical to the enrichment reactor. An experiment lasted for 24 h, from the moment that the reactors were seeded with effluent of a PHB producing enrichment. Each reactor was filled with 700 mL of a mixture of medium and inoculum, the content of the mixture is elaborated later in this section. The reactors were aerated using a fine bubble dispenser at a flow rate of 200 mL min^{-1}, and stirred at 750 rpm to ensure mixing and no oxygen limitation. The reactors were kept at 30 ± 1 °C by a warm water jacket on the outside of the reactor. There was chosen to use a feed-on-demand feeding regime to mimic a feeding regime that can be applied in a full-scale situation. In the beginning of the experiment alkalinity was generated by addition of a dose of sodium acetate. The pH of the reactor broth was monitored and adjusted to 7.0 ± 0.5 by feeding of substrate (elaborated below) which contained amongst others acetic acid. Acetate consumption elevated the pH and thus activating pH control creating a feed-on-demand feeding regime. When the culture is for example saturated with PHA no more acetic acid will be consumed and thus no more elevation of the pH. This created a self-regulating system and would automatically stop feeding substrate when the culture was saturated with PHA.

For the nitrogen limited (N-limited) experiments, the substrate mixture contained 200 mL mixed effluent of the inoculum reactor, 1.0 mM KH_2PO_4, 0.78 mM $MgSO_4$, 1.0 mM KCl, and 5.3 mL of a trace element solution (Vishniac and Santer, 1957). For the phosphorus limited (P-limited) systems the substrate mixture contained 200 mL mixed effluent of the inoculum reactor, 27 mM NH_4Cl, 0.78 mM $MgSO_4$, 1.0 mM KCl, and 5.3 mL of a trace element solution (Vishniac and Santer, 1957).

Subsequently, each bioreactor got a designed substrate, according to the specifications summarized in Table 1. The substrates were designed to produce 2 $gX{\cdot}L^{-1}$ of biomass with a PHB wt% according to the amount of excess carbon. One design substrate bottle will be elaborated in more detail, the rest of the substrate compositions were designed using a similar procedure and shown in Table 1.

The substrate composition for a system designed to reach N-limited conditions and simultaneously 0.80 $gPHB{\cdot}gVSS^{-1}$ will be elaborated. The first design parameter set was the amount of biomass to be produced, this was set to 2 $gX{\cdot}L^{-1}$ (X = biomass). This concentration was chosen so no oxygen limitation should occur. Next, a molecular formula of $C_1H_{1.8}O_{0.5}N_{0.2}$ with a molar mass of 25.1 g mol^{-1} was assumed, so the 2 $gX{\cdot}L^{-1}$ equals 79.6 $mmolX{\cdot}L^{-1}$ (Beun et al., 2002; Metcalf and Eddy, 2003). There is 0.2 mol nitrogen per carbon mole of biomass, thus 15.9 $mmolN{\cdot}L^{-1}$ is required to produce 2 $gX{\cdot}L^{-1}$. The amount of acetate required for growth was calculated as follows (Eq. (1)):

$$\mathrm{Ac\ (growth)} = \frac{X}{MW_X}\cdot Y^{\frac{PHB}{X}}\cdot Y^{\frac{Ac}{PHB}}(\mathrm{accumulation}) \quad (1)$$

- Ac (growth) ($mol{\cdot}L^{-1}$) is the amount of acetate required to produce biomass in the accumulation experiment
- X ($gX{\cdot}L^{-1}$) is the designed biomass concentration in the accumulation experiment (2 $gX{\cdot}L^{-1}$)
- MW_x (gX mol^{-1}) is the molecular weight of biomass (25.1 gX mol^{-1} (Beun et al., 2002))
- $Y^{PHB/X}$ ($Cmol{\cdot}Cmol^{-1}$) is the amount of PHB required to produce 1 Cmol of biomass (1.5 $CmolPHB{\cdot}CmolX^{-1}$ (Marang et al., 2013))
- $Y^{Ac/PHB}$ (accumulation) ($Cmol{\cdot}Cmol^{-1}$) is the amount of acetate required to produce 1 Cmol of PHB (1.2 $Cmol_{acetate}{\cdot}Cmol^{1}_{PHB}$ slightly lower than the reported value of 1.5 $Cmol_{acetate}{\cdot}Cmol^{1}_{PHB}$ was assumed (Jiang et al., 2011b; Marang et al., 2013)). The yield was chosen slightly lower as the yield was reported to decrease over time in an accumulation (Tamis et al., 2018).

With the biomass concentration set at 2 $gX{\cdot}L^{-1}$, Ac(growth) will be 99.1 $mmol_{Acetate}{\cdot}L^{-1}$. All other growth-nutrients were supplied in the

Table 1
Overview of the substrate compositions used in this study.

Name	Target PHB wt%	Ammonium mmolN	Phosphate mmolP	Acetate mmol
CODN-50	90	16	8.4	797
CODN-26	80	16	8.4	409
CODN-13	60	16	8.4	215
CODN-9	40	16	8.4	151
CODP-996	90	32	0.8	797
CODP-511	80	32	0.8	409
CODP-269	60	32	0.8	215
CODP-189	40	32	0.8	151

same acetate to nutrient ratio as in the enrichment reactor (Marang et al., 2013). Ac (growth) was taken to determine the amount of nutrients required for growth. Another design parameter was that the culture should reach and maintain a PHB percentage of 80 wt% PHB. The acetate required for PHB production was obtained as follows (Eq. (2)):

$$\text{Ac (PHB)} = \frac{\text{X}\cdot\text{PHB wt\%}}{100 - \text{PHB wt\%}}\cdot \text{MW}_{\text{PHB}}\cdot \text{Y}^{\frac{\text{Ac}}{\text{PHB}}}(\text{accumulation}) \quad (2)$$

- Ac (PHB) ($\text{mol}\cdot\text{L}^{-1}$) is the amount of acetate required to produce the target PHB wt%
- PHB wt% is the designed target PHB wt%
- MW_{PHB} ($\text{g}\cdot\text{Cmol}^{-1}$) is the molecular weight of PHB (21.5 $\text{g}\cdot\text{Cmol}^{-1}$ (Beun et al., 2002))

A target of 80 wt% PHB results in that Ac (PHB) is 310 $\text{mmol}\cdot\text{L}^{-1}$. In total 409.1 $\text{mmol}\cdot\text{L}^{-1}$ is required to produce 2 $\text{gX}\cdot\text{L}^{-1}$ with a PHB content of 80 wt%. A similar approach was used to obtain the other designed substrates.

2.2.1. Sampling and analytical methods

During all fed-batches a sample was withdrawn every 3 h. Samples were analysed for PHB content, total suspended solids (TSS), volatile suspended solids (VSS), acetate, NH_4^+-N and PO_4^--P concentrations. Samples withdrawn from the reactor that were analysed for (NH_4^+, PO_4-, acetate) were directly after sampling filtered using a 0.45 μM PVDF filter (0.45 μm pore size, PVDF membrane, Millipore, Ireland). Soluble samples were analyzed using a Gallery™ Plus Discrete Analyzer (Thermo-Fischer Scientific), commercially available. TSS and VSS samples were measured according to standard methods (Clesceri et al., 1999), and PHB measurements were performed on dried samples according to a method supplied in an earlier study (Johnson et al., 2009).

2.2.2. Microbial community analysis

Fluorescent In Situ Hybridization (FISH) was used to analyse the microbial community composition. The probes used were a mixture of the probes UCB823 and EUB338I-III. A more detailed version of performing this FISH technique can be found in previous work (Johnson et al., 2009). Commercially synthetized probes with either 5′ sulfoindocyanine dyes Cy5 and Cy3 (Thermo Hybrid interactive, Ulm, Germany) were used, summarized in Table 2.

3. Results

In this study two sets of fed-batch experiments were conducted using for each set an enrichment culture from the same steady-state reactor. Operational procedures of the enrichment reactor are described in the materials and methods section. The enrichment was dominated by *Plasticicumulans acidivorans* as determined using FISH microscopy after running a test-accumulation. Following Fig. 1 an estimated dominance of *P. acividorans* of 90 % or higher was observed.

Four separate bioreactors were used for PHA accumulation experiments at different degrees of nitrogen limitation and four separate bioreactors for phosphorus limitation experiments. The experiments were operated for 24 h. A complete overview of the collected data can be found in the supplementary materials (Appendix A). Two representative datasets for N and P limitation were selected to be elaborated in more detail: 1) the experiment designed to be P-limited and reaching 80 wt% PHB (CODP-511) and 2) the experiment designed to be N-limited and reaching 80 wt% PHB (CODN-26). Nutrients supply with the inoculum was minimized by lowering the ammonium or phosphate dosage in the enrichment reactor the cycle before the experiment was conducted (see the materials and methods section).

Table 2
Oligonucleotides probes used for FISH analysis in this study.

Code	Sequence (5′ -3′)	Specificity	Reference
EUB338 I	gctgcctcccgtaggagt	Bacteria	(Amann et al., 1990)
EUB338 II	gcagccacccgtaggtgt	Bacteria	(Daims et al., 1999)
EUB338 III	gctgccacccgtaggtgt	Bacteria	(Daims et al., 1999)
UCB823	cctccccaccgtccagtt	*P. acidivorans*	(Johnson et al., 2009)

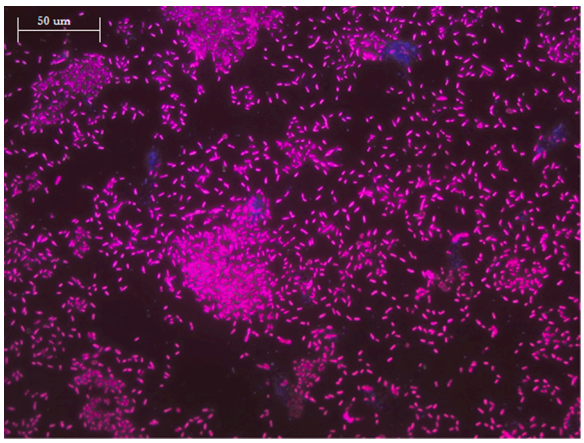

Fig. 1. FISH picture of PHA enriched culture during a test-accumulation. Stained with Cy3-labeled UCB823 Red (*Plasticimulans acidivorans*) and Cy5-labeled EUB338 (Eubacteria) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.1. Phosphorus limited PHB accumulation targeting 80 wt% PHB (CODP-511)

An experiment was started by supplying a dose of acetate to the reactor to achieve a concentration of 27 mM. After this a feed-on-demand regime (fed-batch) was created as the pH controlling agent was the feed. A feed-on-demand pattern was chosen to have carbon substrate in access at all time. Within 3 h the culture reached the target 80 wt% PHB, and maintained the target PHB wt% of 80 % (within a margin of 5%-point) for 20 h before dropping to 60 wt% PHB. The overall PHA production yield varied over time but remained around 0.54 ± 0.15 $\text{gCOD}_{\text{PHA}}\cdot\text{gCOD}_{\text{Acetate}}^{-1}$ (n = 7; average ± standard deviation), before the yield lowered to 0.12 $\text{gCOD}_{\text{PHA}}\cdot\text{gCOD}_{\text{Acetate}}^{-1}$ from h 20 to 24.

In this accumulation experiment the measured phosphate concentration was continuously below the detection limit of 0.01 mg $\text{P}\cdot\text{L}^{-1}$. The substrate specific uptake rate expressed as $\text{gCOD}_{\text{Acetate}}\cdot\text{gX}^{-1}\ \text{h}^{-1}$ (X = biomass measured as VSS minus PHB) was high for the first 5 h of the experiment after which it stabilized around 1–2 $\text{gCOD}_{\text{Acetate}}\cdot\text{gX}^{-1}\ \text{h}^{-1}$. After the initial 5 h of the experiment biomass growth was detected and the biomass production yield increased shown in Fig. 2. Until 20 h the measured acetate concentration was above 400 $\text{mgCOD}\cdot\text{L}^{-1}$, the concentration of acetate lowered to 120 $\text{mgCOD}\cdot\text{L}^{-1}$ at 24 h.

3.2. Nitrogen limited PHB accumulation with a target of 80 wt% (CODN-26)

For the second set of accumulation experiments a similar approach was used to initiate the feed-on-demand pattern as discussed before, after initial addition of acetate to the system. The culture reached within 9 h the target PHB wt% of 80 % ± 5%-point and maintained this PHB wt % for an additional 9 h before dropping to 70 wt% (Fig. 3). The observed PHB yield until 18 h was 0.64 ± 0.15 $\text{gCOD}_{\text{PHA}}\cdot\text{gCOD}_{\text{Acetate}}^{-1}$ (n = 6), afterwards the PHB wt% started to decline.

During the entire experiment the extracellular NH_4^+ concentration was below the detection limit of 0.005 mg $\text{N}\cdot\text{L}^{-1}$. In this experiment the substrate specific uptake rate rapidly decreased in the first 4 h. At the

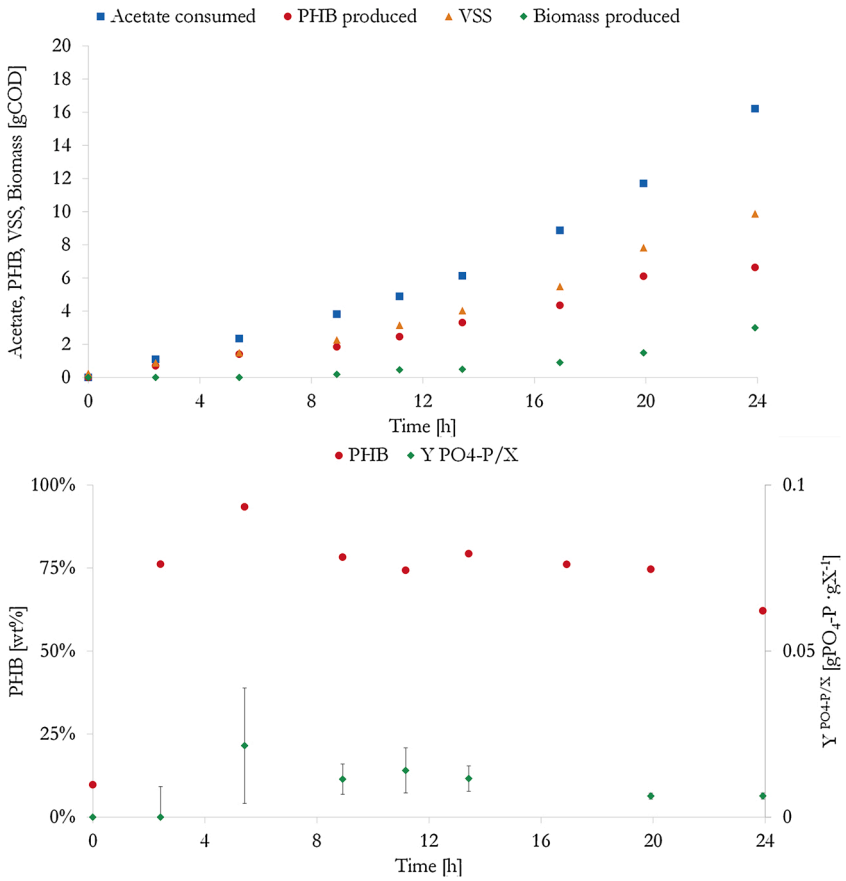

Fig. 2. Phosphorus limited accumulation targeting 80 wt% poly(3-hydroxybutyrate) (PHB) (CODP-511). The top graph shows the acetate consumed, PHB produced, total amount of volatile suspended solids (VSS) present, biomass produced, all in Chemical Oxygen Demand (COD) equivalents. The bottom graph shows PHB wt% and the yield of NH4-N per X produced with a 95 % confidence interval.

start of the fed-batch the $q_{Acetate}$ was around 4.5 $gCOD_{Acetate} \cdot gX^{-1}\ h^{-1}$. This $q_{Acetate}$ decreased after the first 4 h and was for the rest of the experiment 0.80 ± 0.31 (n = 7) $gCOD_{Acetate} \cdot gX^{-1}\ h^{-1}$. The measured acetate concentration was for the duration of the experiment 50 $mgCOD \cdot L^{-1}$ or higher.

3.2.1. *Overview of the results*

In all experiments the designed nutrient limitation occurred within 6 h and was limited for the remainder of each experiment. In all experiments the acetate concentration was at all times 50 $mgCOD \cdot L^{-1}$ or higher and the highest measured acetate concentration was around 1700 $mgCOD \cdot L^{-1}$. The system performance was analysed using the PHB wt% of the culture to evaluate whether the target PHB wt% was reached and maintained.

Generally, the PHB target wt% (± 5%-point) was reached within 6 h for all systems and the target was maintained for at least an additional 6 h. In all the N-limited systems no PHB wt% decline larger than 10 %-point compared to the target was observed. In all P-limited systems, a larger than anticipated decline in PHB wt% was observed after 13 h for CODP-189 and CODP-269 and after 20 h for the CODP-511 system.

Despite the similar initial amount of biomass (X_0) in the N-limited systems, the volumetric PHB productivity ($gPHB \cdot L^{-1}\ h^{-1}$) was in general higher in the P-limited systems compared to the N-limited systems. The higher productivity in the P-limited system concurred with the higher amount of active biomass that was produced in these systems during the experiment. This is also reflected in the $Y^{\frac{NH_4-N}{X}}$ (Fig. 3) which remained relatively constant over time in contrast to $Y^{\frac{PO_4-P}{X}}$ (Fig. 2) which slightly lowered over time. This indicated that in the P-limited systems over time more X could be produced per PO_4-P consumed.

In order to assess the stability of the system a variable named $t_{optimal}$ was defined. $t_{optimal}$ was the time point in an experiment when the PHB content deviated more than 5%-point below the target PHB wt% for at least two measurements (Table 3). All experiments maintained their target PHB wt% at least until 12 h before reaching $t_{optimal}$. As example in the CODP-996 system the PHB wt% was 0.87 $gPHB \cdot gVSS^{-1}$ at 17 h, the following two measurement both were below 0.85 $gPHB \cdot gVSS^{-1}$ making $t_{optimal}$ 17 h.

4. Discussion

Wastewaters from all kind of industries are often not strictly growth nutrient limited and this can affect the overall bioplastic production possibilities. Hence, in this study we investigated the production of PHB at different degrees of P- and N-limitation. The initial hypothesis was that the PHB wt% of a culture could be predicted based on the C/N ratio or C/P ratio in the substrate. To test to which extent this hypothesis holds true and for how long the predicted PHB content can be maintained, 8 fed-batch experiments were performed. In the systems targeting lower PHB wt%, e.g. 40 % and 60 %, initially after the start-up of the experiment PHB contents exceeded the predicted PHB wt% for 6−12 h. After this period the PHB wt% decreased to the designed target wt%. The minimum PHB wt% could be accurately predicted based upon on the C/N and C/P ratio for at least 12 h.

4.1. *Nitrogen limited PHB accumulation and growth*

In the N-limited systems the predicted PHB percentages were achieved for at least 12 h before reaching $t_{optimal}$, the timestamp in an experiment from which the culture started to negatively deviate more

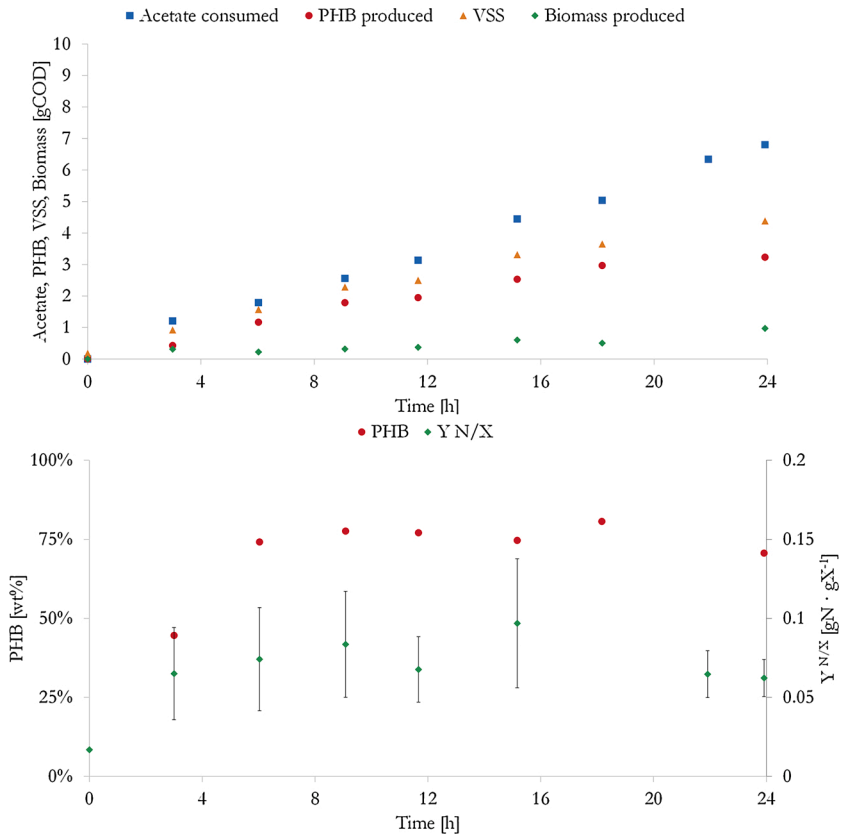

Fig. 3. Nitrogen limited accumulation targeting 80 wt% poly(3-hydroxybutyrate) (PHB) (CODN-26). The top graph shows the acetate consumed, PHB produced, total amount of volatile suspended solids (VSS) present, biomass produced, all in Chemical Oxygen Demand (COD) equivalents. The bottom graph shows the PHB wt % and the yield of PO4-P per X produced with a 95 % confidence interval.

Table 3
System characteristics obtained for each system at $t_{optimal}$. $t_{optimal}$ was defined as the time in an experiment when the PHB wt% of the culture fell below the target PHB wt% for at least 2 measurements in a row. X stands for biomass.

System	$t_{optimal}$ h	PHB gPHB·gVSS^{-1}	$Y^{PHA/Acetate}$ gCOD·gCOD^{-1}	$Y^{X/Acetate}$ gCOD·gCOD^{-1}
CODP-996	17	0.87	0.68 ± 0.27 (n=6)	0.07 ± 0.10 (n=4)
CODP-511	20	0.75	0.54 ± 0.15 (n=7)	0.12 ± 0.11 (n=4)
CODP-269	13	0.61	0.55 ± 0.17 (n=3)	0.21 ± 0.17 (n=3)
CODP-189	13	0.35	0.36 ± 0.55 (n=5)	0.47 ± 0.34 (n=4)
CODN-50	12	0.85	0.66 ± 0.20 (n=2)	0.11 ± 0.23 (n=3)
CODN-26	18	0.81	0.64 ± 0.37 (n=6)	0.05 ± 0.18 (n=6)
CODN-13	22	0.59	0.52 ± 0.33 (n=7)	0.24 ± 0.15 (n=7)
CODN-9	24	0.42	0.20 ± 0.31 (n=7)	0.33 ± 0.28 (n=7)

than 5%-point from the target PHB wt%. Comparable results were obtained in previous work using a *P. acidivorans* culture in a single N-limited accumulation (Johnson et al., 2010a). Johnson et al. (2010a) conducted an accumulation experiment at a C/N ratio targeting 60−80 wt% PHB and obtained a PHB content of 77 wt% after 9.6 h. In the current work and in Johnson et al. (2010a) a designed medium resulted in different targeted PHB wt% combined with biomass growth. The PHB wt% were predictable from the composition of the substrate, i.e. the C/N ratio. Remarkably, $t_{optimal}$ seemed to be related to the degree of limitation. At lower C/N ratios $t_{optimal}$ was reached later, as $t_{optimal}$ was 24 h for CODN-9 versus 12 h for CODN-50. The production of PHA is a widespread trait throughout the microbial world, however reaching high PHA wt% can only be achieved by specialists (Kourmentza et al., 2017). Reaching lower wt% PHB (<60 wt%) can be done by microorganisms that are present in the activated sludge process from a municipal wastewater treatment plant (Werker et al., 2018). In the CODN-9 and CODN-13 the amount of PHB that the side-population needs to produce can be lower than the target as the culture likely exist of a mixture of *Plasticicumulans* able to reach higher than 60 wt% PHA. The combination of having a PHB specialist with a non-specialist both capable of producing PHB can explain why $t_{optimal}$ was reached later for lower PHB wt% targets. Additionally, the biomass concentration increased significantly in the CODN-9 systems as 12 times the initial biomass amount was present at 24 h. No significant increase in the substrate uptake rates were observed despite the biomass growth (data shown in supplementary materials). This implied that exponential growth did not occur in the N-limited systems, otherwise an increased substrate uptake rate would have been observed.

4.2. Phosphorus limited PHB accumulation and growth

The overall performance of the phosphorus limited systems after $t_{optimal}$ was different from the nitrogen limited systems i.e. after $t_{optimal}$ the PHB content dropped faster. Furthermore, the P-limited experiments could roughly be divided in 2 groups namely, the first group CODP-996 and CODP-511 which reached $t_{optimal}$ relatively late (17−20 h) and CODP-269 and CODP-189 reaching $t_{optimal}$ relatively early (13 h). The lower cellular PHB content after $t_{optimal}$ may indicate two phenomena: (i) the P-content of the biomass was lowered under P-limited conditions, or (ii) a side-population specialized in phosphate uptake starts proliferating in the system at the expense of a decreasing contribution of *P. acidivorans*. If the P-content per unit of biomass was lowered, more active biomass (as VSS) can be produced per phosphate consumed as proposed by Korkakaki et al. (2017). The decrease in the P-content of active biomass therewith results in an increase in the biomass yield on substrate and consequently a decrease in PHA yield and a lower PHB wt %. Finally, a combination of the abovementioned phenomena may have

occurred, resulting in a lower PHB content per cell. The phenomenon of adaptation of the phosphorus content of *P. acidivorans* was previously investigated and it was shown that the cellular P-content of *P. acidivorans* was reduced under P-limiting conditions (Korkakaki et al., 2017). The dilution of the phosphorus content per cell did not affect the PHB accumulating capacity as a high PHB potential was obtained, when accumulating under growth restricted conditions. In this study the effects of the possible P-dilution occurred late as the earliest observed $t_{optimal}$ for the P-limited experiments was 13 h.

4.3. Overall system performance

The PHB accumulating potential was evaluated by looking at the PHB production yield at $t_{optimal}$. In general, a higher PHB production yield was obtained at higher PHB wt%. Higher PHB production yields were obtained at higher target PHB wt% as more carbon was allocated to PHB production per biomass-unit produced.

Overall, in our study, the final PHB content could adequately be predicted based on the C:N or C:P ratios of the feed. To achieve a high PHB cellular content (>75 PHB wt%) the carbon to nutrient ratio should be high. This observation showed the sensitivity of the accumulation process, and the requirement of using a nutrient-limited (waste)stream to reach a high PHB cellular content regardless of the inoculum.

4.4. Outlook

The production of PHA from wastewater using mixed microbial cultures has been studied for over 10 years. Conditions have been identified that enable the enrichment of a superior PHA producing microbial community using a feast-famine regime operated at a short SRT of 1 d and a cycle length of 12 h (Johnson et al., 2009). Later this PHA specialist was isolated and annotated as *Plasticiumulans acidivorans*, and is now a thoroughly studied microorganism (Jiang et al., 2011b). The feast-famine setting resulting in the enrichment of a PHA specialist also have been successfully applied on real wastewater streams such as the candy bar factory or the paper mill factory (Tamis et al., 2018, 2014). Tamis et al. (2014) found that enriching on fermented candy bar wastewater (containing VFA and other COD) resulted in PHA producing microorganisms and a side-population. The effect of this non-VFA COD on the enrichment step was studied in the lab (Korkakaki et al., 2016b; Marang et al., 2014). These studies demonstrated that enrichments supplied with VFA will result in the PHA producing specialist *P. acidivorans* and non-VFA (methanol) will not result in this PHA producing specialist.

One of the next challenges encountered was that PHA production from the leachate of the organic fraction of municipal solid waste (OFMSW) seemed challenging due to enrichment difficulties (Korkakaki et al., 2016a). One approach to circumvent enrichment difficulties applied by Korkakaki et al. (2016a) was to supply the leachate of the OFMSW to a *P. acidivorans* culture that had been established on synthetic medium. That approach is comparable to the work described in this paper since in both studies the *P. acidivorans* enrichment was fed with a VFA-rich stream containing growth-nutrients.

The results in this study can help facilitate the waste-based production of PHA. It was observed that if the substrate contained a ratio of CODP > 511 and/or a CODN > 26 a high PHA wt% (PHA wt% > 75 %) can be maintained for at least 12 h. This indicates that operating the accumulation can be less stringently as an operational window of at least 12 h exist before the PHA wt% will drop. On the contrary, if the substrate contained more nutrients for example in experiment CODN-13 the highest observed PHA wt% was 0.71 gPHA·gVSS at t =9 h (shown in the supplementary material). The consequence of this observed PHA_{max} is that likely operating the accumulation is more challenging as the window of reaching the highest possible PHA purities is narrow.

Additional research to elucidate to an even larger extent the effect of nutrients on the accumulation are: (i) performing the same experiments as done in this work though using a higher degree of enriched culture (>99 % presence) of *P. acidivorans* to see whether the same effects will be observed. (ii) Using different feeding patterns, in this study the feed was a continuous stream containing both carbon source and nutrient source. It could be interesting to find out whether a culture can maintain longer its PHA producing capability by altering the feeding pattern. For example, when the carbon feed is separated from the nutrient feed, the carbon feed can still be continuous and the nutrient feed can be dosed pulse-wise. Potentially, in this way it can be circumvented that a specialist for nitrogen or phosphorus uptake will take over the reactor at e.g. CODN-13. Overall, the production of PHA is nowadays possible from a wide variety of (waste)streams using a range of different strategies.

5. Conclusion

Not all waste streams are strictly growth nutrient limited. The effect of these nutrients on the overall PHA production process was in more detail investigated in this work. In this study we investigated the effect of N- and P-limitation on the PHA accumulation potential using a pre-enriched *P. acidivorans* dominated culture. 8 accumulation experiments in a multi-reactor set-up were performed with different ratios of excess carbon and 1 growth nutrient either nitrogen or phosphorus designed to be limited. Experimental results demonstrated that for at least 12 h the PHB wt% (higher or 5%-point lower) could be predicted based upon the substrate composition, i.e. the carbon to nutrient ratio. This study showed that the presence of small amounts of nutrients did not hamper the PHA production process as high PHA purities were maintained for an extended period of time.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btecx.2020.100027.

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