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Estévez-Alonso, Ángel; Altamira-Algarra, Beatriz; Arnau-Segarra, César; van Loosdrecht, Mark C.M.; Kleerebezem, Robbert; Werker, Alan

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Process conditions affect properties and outcomes of polyhydroxyalkanoate accumulation in municipal activated sludge

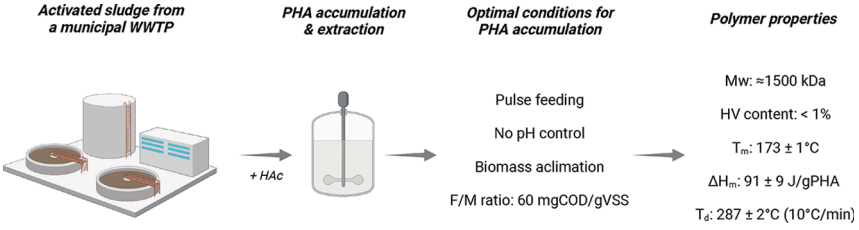
Ángel Estévez-Alonso [a,b,*], Beatriz Altamira-Algarra [b], César Arnau-Segarra [b], Mark C.M. van Loosdrecht [a], Robbert Kleerebezem [a], Alan Werker [b]

[a] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands
[b] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911, MA, Leeuwarden, The Netherlands

HIGHLIGHTS

- Pulse feeding favored PHA production compared to excess substrate supply.
- An acclimation step combined with pulse feeding resulted in higher PHA contents.
- PHA molecular weights in the order of 1500 kDa were obtained.
- Extractable PHA was influenced by its molecular weight and melt properties.
- A standard protocol for PHA accumulation tests with activated sludge was developed.

GRAPHICAL ABSTRACT



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ABSTRACT

The developments of mixed culture polyhydroxyalkanoate production has been directed to maximize the biomass PHA content with limited attention to polymer quality. Direct comparison of PHA accumulation literature is challenging, and even regularly contradicting in reported results, due to underlying differences that are not well expressed. A study was undertaken to systematically compare the commonly reported process conditions for PHA accumulation by full-scale municipal activated sludge. A biomass acclimation step combined with a pulse-wise feeding strategy resulted in maximum average PHA contents and product yields. pH control and active nitrification did not result in observable effects on the PHA productivity. Under these conditions a high molecular weight polymer (1536 ± 221 kDa) can be produced. Polymer extraction recoveries were influenced by the PHA molecular weight. A standard protocol for an activated sludge PHA accumulation test including downstream processing and standardized extraction has been developed and is available as supplementary material.

1. Introduction

Waste activated sludge is produced as a by-product in biological wastewater treatment plants (WWTP). Its production, and subsequent disposal, contributes significantly to WWTP operational costs (Appels et al., 2008). Within regionally based circular economies, waste activated sludge does not necessarily need to be a waste (Tyagi and Lo, 2013). Municipal waste activated sludge can instead be a resource for the production of alginate-like exopolysaccharide or polyhydroxyalkanoates (PHAs) (Kehrein et al., 2020). It can be a raw material input for a PHA production process if volatile fatty acid (VFA) rich streams can be made sufficiently available as feedstock (Bengtsson et al.,

* Corresponding author at: Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
E-mail address: a.estevezalonso@tudelft.nl (Á. Estévez-Alonso).

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2017).

PHAs are intracellularly stored bacterial energy and carbon reserves that can be extracted from biomass and applied in the polymer industry (Philip et al., 2007). PHAs represent an alternative to non-biodegradable oil-based polymers, but the current levels of industrial production are not able to compete economically with the traditional oil-based polymer industry (Estévez-Alonso et al., 2021). PHA production methods are evolving to meet ongoing goals of increased production capacity with improved economy. Waste activated sludge from a biological WWTP and fermented organic waste and wastewater streams can be used for PHA production. Waste activated sludge can be used directly for PHA production or used as inoculum for the enrichment of a PHA-storing biomass (Estévez-Alonso et al., 2021). The *enrichment* strategy has been investigated over two decades (Estévez-Alonso et al., 2021; Valentino et al., 2017). However, a *direct* accumulation approach has received less research attention (Arcos-Hernandez et al., 2013).

Considerable efforts at lab and pilot scales have been directed to establish optimal operational conditions that maximize the biomass PHA content. Examples of the operational conditions that have been studied until now are: the modulation of nutrient concentrations in the feedstock (Silva et al., 2017; Valentino et al., 2015; Johnson et al., 2010; Punrattanasin et al., 2006; Wen et al., 2010; Chinwetkitvanich et al., 2004; Cavaillé et al., 2013), redox conditions (Liu et al., 2011; Pratt et al., 2012; Punrattanasin et al., 2006; Moralejo-Gárate et al., 2013), the feeding composition and feeding strategy (Serafim et al., 2004; Palmeiro-Sánchez et al., 2016; Albuquerque et al., 2011; Valentino et al., 2015; Chen et al., 2013; Ma et al., 2000; Chen et al., 2015), temperature (De Grazia et al., 2017; Johnson et al., 2010; Chinwetkitvanich et al., 2004), pH control and salinity (Palmeiro-Sánchez et al., 2016; Mulders et al., 2020; Chen et al., 2013), presence or absence of nitrifying bacteria (Fra-Vázquez et al., 2019), and different biomass pretreatments (Morgan-Sagastume et al., 2017). Direct comparison between these studies is challenging due to the differences in the selected PHA-storing biomass and applied process conditions for PHA accumulation. For instance, studies on the influence of nutrients on the accumulation of PHA have shown contradictory results. Johnson et al. (2010) reported that maximum expressed cellular PHA content required the absence of nutrients in the feedstock. To the contrary, Valentino et al. (2015) found higher expressed PHA content when nutrient limiting conditions in the feedstock were applied. In these two studies, the PHA-storing biomass was enriched at different temperature, solid retention times and cycle length and furthermore, in the PHA accumulation reactor the feeding strategy, temperature and pH range were also different. This example illustrates contradicting outcomes and suggests that underlying context is an important factor. Most of these studies have also used specifically enriched biomass for the PHA accumulation, in part, to assess for the influence of the selection pressure on the biomass production and quality. Systematic evaluations for PHA accumulation with waste activated sludge, without further enrichment, are lacking in the research literature.

Optimal outcomes for industrial PHA production are a maximal quantity with consistent quality. Mixed culture PHA production typically produce co-polymer blends of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or PHBV. Most of the studies mentioned above have focused on the maximization of the biomass PHA content without evaluation of quality other than the average co-polymer composition (Silva et al., 2017; Palmeiro-Sánchez et al., 2016; Albuquerque et al., 2011). Few studies have explored how accumulation conditions can influence the polymer quality and/or consistency of quality (Lorini et al., 2021; Werker et al., 2020; Laycock et al., 2013). Polymer quality relates to the polymer mechanical and thermal properties. Polymer mechanical and thermal properties are affected by the co-polymer composition and the polymer molecular weight. The co-polymer composition is mainly determined by volatile fatty acids composition in the feedstock, that would result in the production of the monomer 3-hydroxyvalerate (3HV) or 3-hydroxybutyrate (3HB) (Werker et al., 2020). Polymer thermal properties are directly related to the average 3-hydroxyvalerate content assuming a random monomer distribution in the blend. However, control of the polymer molecular weight outcomes are poorly understood with few publications on the subject (Serafim et al., 2008; Bengtsson et al., 2010; Albuquerque et al., 2011; Palmeiro-Sánchez et al., 2016; Bengtsson et al., 2017; Lorini et al., 2021; Werker et al., 2020; Lorini et al., 2021; Rodrigues et al., 2022).

The aim of this work was to compare selected commonly reported process conditions for PHA accumulation using full-scale municipal waste activated sludge as the PHA-producing biomass. Principal factors identified from the research literature were biomass acclimation, feeding strategy, pH control and, presence of nitrifying bacteria. Polyhydroxybutyrate was produced with acetic acid to enable a study focused on how accumulation conditions influenced outcomes of polymer quantity and molecular weight quality. As a result, a standard protocol for an activated sludge PHA accumulation test including downstream processing and standardized extraction has been developed and is available as supplementary material.

2. Materials and methods

2.1. Experimental set-up

PHA accumulation tests were performed over 24 h in a 1 L double-jacketed glass bioreactor at 25 ± 0.1°C. In cases with pH control (Table 1), pH was maintained at 7.5 ± 0.1 with feedback control dosage of 1 M HCl. The airflow rate was fixed at 1 L/min (MV-302, Bronkhorst, Germany) and agitation at 150 rpm was by standard three-bladed turbine (R60, CAT Scientific, Germany). Dissolved oxygen and pH probes (COS81D and CPS11D, Endress & Hausser, The Netherlands) were coupled to a 4-channel transmitter (Liquiline CM444, Endress & Hausser, The Netherlands) and measurements were logged every 10 s. Probes were calibrated according to manufacturer instructions for each assay. Substrate feed dosing diaphragm pumps (Stepdos 10, KNF, The Netherlands) were actuated by PLC (Logo! 8 and Logo! TDE, Siemens, Germany).

2.2. Sludge source and feedstock

Waste activated sludge from the municipal WWTP Bath (Rilland-Bath, The Netherlands) was used for PHA accumulation. Fresh gravity belt thickened waste activated sludge (56 ± 4 gTS/kg and 39 ± 3 gVS/kg) was delivered batch wise by courier every two weeks in 20 L carboys and stored at 4°C pending experiments. In total, 14 distinct batches delivered over a period of six months were used for these experiments.

The accumulation feedstock, with nutrients ratio 100:1:0.05 (COD:N:P by weight), was prepared with tap water as follows: 50 g/L acetic acid, 1.91 g/L NH_4Cl, 109.6 mg/L KH_2PO_4. The feedstock pH was adjusted to 5.5–6 with KOH pellets.

2.3. PHA accumulations with different process conditions

A total of 25 PHA accumulation experiments were performed to evaluate an influence of process conditions (Table 1). For each experiment, an aliquot of the stored activated sludge was diluted with tap water to nominally 2.5 gVSS/L. The biomass was conditioned at 25 ± 0.1°C by aeration overnight to establish a baseline of endogenous microbial activity. Allylthiourea (20–50 mg/L) was then added directly to the reactor in some cases (Table 1). The air supply was interrupted for 30 min to assess the endogenous respiration level. The subsequent re-aeration period was used to determine the oxygen mass transfer coefficient k_La. PHA accumulation was then started according to pre-selected protocols of feeding. The feedstock solution was dosed continuously or in fixed-volume pulses to target a selected maximum peak substrate concentration of 50, 150 or 1000 $mgCOD_{HAc}/L$. These targeted substrate concentrations were chosen to reach an initial food to microorganism

Table 1
Overview of the accumulation tests and the operational conditions used. F/M ratio = initial food to microorganism ratio; ATU = allylthiourea.

Test -	Feeding -	Pulse F/M ratio $mgCOD_{HAc}/gVSS$	pH control -	ATU -	Acclimation -	N° of tests -
1	Pulse-wise	20	Yes	Yes	No	3
2	Pulse-wise	60	Yes	Yes	No	4
3	Pulse-wise	60	Yes	No	No	3
4	Pulse-wise	60	No	No	No	4
5	Pulse-wise	60	No	No	Yes	5
6	Pulse-wise	400	Yes	Yes	No	3
7	Continuous	200	Yes	Yes	No	3

ratio (F/M ratio) of 20, 60 and 400 $mgCOD_{HAc}/gVSS$, respectively. Continuous excess feeding was started with a pulse of 500 $mgCOD_{HAc}/L$ and with subsequent continuous substrate addition. The rate for substrate addition in the continuous excess feeding was based on the average rate of substrate consumption from previous experiments. The continuous excess feeding aimed to reach a food to microorganism ratio of 200 $mgCOD_{HAc}/gVSS$. Fixed-volume pulse inputs were controlled from on-line monitoring of dissolved oxygen concentrations, as previously reported (Valentino et al., 2015; Werker et al., 2013a). PHA accumulation tests with fixed-volume pulses of 150 $mgCOD_{HAc}/L$ were also used to investigate for an influence of pH control at 7.5, presence of active nitrifying bacteria, and short-term biomass acclimation. For acclimation, the biomass was subjected to three feast and famine cycles before the start of the accumulation process (Morgan-Sagastume et al., 2017). Feast conditions were generated with a pulse input to reach a maximum substrate level of 150 $mgCOD_{HAc}/L$ and the duration of the feast phase was monitored by dissolved oxygen concentration. The applied famine period was three times longer than the feast time. After the third famine period, the accumulation feed-on-demand process was started automatically.

2.4. Biomass downstream processing and polymer extraction

At the end of the experiment, the reactor mixed liquor was collected and acidified to pH 2 with 95% H_2SO_4. Suspended solids were recovered after centrifugation (10000 rcf and 4°C for 20 min). Subsequently, the recovered wet PHA-rich solids were heated at first for 30 min at 120°C followed by overnight drying at 105°C. PHA quantity and quality in the dried biomass were assessed by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and pyrolysis with gas chromatography combined with mass spectroscopy (PyGCMS) (see analytical methods).

The polymer was extracted in 13 mL dimethyl carbonate (DMC, Sigma–Aldrich ReagentPlusö, 99%) from weighed ground biomass samples to target a maximum theoretical polymer concentration of 20 mgPHA/mL. Ground samples were first weighed and re-dried for 30 min at 105°C in tare weighed 20 mm diameter) glass digestion tubes (Hach, LZP065). A weighed mass of DMC was added, and tubes were sealed with respective tare weighed caps. Tube contents were vortex mixed and placed in a 140°C pre-warmed heater block (Hach-Lange, LT200) for 20-min extraction time wherein contents were vortex mixed briefly every 5 min. After the extraction time, and the final vortex mixing, tubes were transferred to an 80°C pre-warmed heater block (Grant, QBD4) and biomass was settled by gravity. About 11 mL of the solvent solution was carefully decanted to pre-warmed (90°C) 15 mL Falcon tubes excluding most suspended solids. The warm solution was centrifuged (up to 9418 x g over 2 min), with pre-heated (90°C) tube inserts to remove remaining suspended solid fines from the polymer solvent solution. A weighed amount (about 10 mL) of the still hot solution was carefully decanted directly into a tare weighed soda-lime glass petri dish (Duran, 90 mm diameter) that was placed on a level drying scale (Satorius MA37). Solvent was evaporated at 85°C with gentle heating and formed a dried solution cast polymer film (< 0.1% mass change over 1 min). Added and removed masses of solids and solvent (± 1 mg) were followed at each step of the extraction protocol for making mass balances. The extracted mass was derived from the solution concentration, estimated from film casting, and the known total mass of extraction solvent used. Extraction yields were calculated relative to the amount biomass in the extraction tube after the initial re-drying before solvent addition. The recovered polymer thermal properties were characterized by TGA and DSC, and weight average molecular mass was estimated by solution rheology (see analytical methods).

2.5. Analytical methods

The PHA accumulation process was monitored by logging DO, pH and temperature. Three 15 mL grab samples were taken at selected times. Suspended solids were separated from the 15 mL of the mixed liquor by centrifugation (3250 rcf and 4°C for 20 min). The supernatant after membrane filtration (0.45 μm pore size filters) was stored at −20°C pending liquid analyses. The harvested biomass pellet dry weight and ash contents were measured according to Standard Methods (105°C drying and 550°C ashing) and referenced to the sample volume for estimated total and volatile suspended solids (TSS and VSS), respectively. Soluble chemical oxygen demand (sCOD) was determined by Hach-Lange (Germany) LCK014 and LCK314 test kits. Acetic acid concentration was determined by ultra-high pressure liquid chromatography and ammonium, nitrite, nitrate and phosphate concentrations were determined by ion chromatography, as previously reported (Estévez-Alonso et al., 2021). 15 mL of mixed liquor were directly acidified to pH 2 with 95% H_2SO_4. After thorough mixing (5 min) suspended solids were collected (3250 rcf and 4°C for 20 min). The biomass pellet was dried at 105°C. Dried pellets were ground by hand with mortar and pestle for polymer characterization before and after extraction.

Thermogravimetric analysis (TGA2, Mettler-Toledo), for PHA content with respect to total solids (TS) and volatile solids (VS) as gPHA/gTS and gPHA/gVS, and for thermal decomposition temperature (T_d), was performed as previously described (Chan et al., 2017). Representative sub-samples of about 5 and 2 mg of dried biomass or recovered polymer were used, respectively. The method includes estimation of sample residual moisture and PHA contents as well as the overall organic and inorganic fractions. Briefly, pre-weighed samples were inserted to the TGA at 80°C with nitrogen purge gas at 50 mL/min. Temperature was increased (10°C/min) to 105°C and held for 15 min wherein moisture (or residual solvent) content could be estimated. Temperature was increased (10°C/min) to 550°C and held for 30 min. PHA mass could be estimated from the characteristic rapid mass loss occurring between 225 and 350°C. At 550°C the purge gas was changed to air at 50 mL/min. Sample ash content was estimated by weight loss at 550°C with air atmosphere. Reference samples included a PHA-rich biomass with known PHA content (45.1 ± 0.6 gPHA/gVS), and pure PHB (> 98% purity, Biomer, Germany). Temperature was calibrated based on Curie temperature with a nickel standard following Mettler-Toledo methods.

Differential scanning calorimetry (DSC 3+, Mettler-Toledo) was also performed based on previously described methods (Chan et al., 2017) and with nominally 5 and 2 mg dried biomass and recovered polymer

samples, respectively. Sample PHA purity was estimated from TGA measurements. Weighed samples were inserted and held for 5 min at −70°C with nitrogen purge gas at 50 mL/min. A first heat and quench cycle followed with heating and cooling at 10°C/min to 185°C and back to −70°C. A second heat ramp at 10°C/min to 185°C was applied followed by quenching (-100°C/min) to −70°C after 0.5-min hold at 185°C. A third heat ramp at 10 °C/min to 185°C was applied followed by quenching (-30°C/min) to −70°C, after 0.5-min hold at 185°C. Finally, the sample was heated at 10°C/min to 40°C. Melt and crystallization enthalpies were estimated with respect to the mass of PHA in the sample. Reference samples included pure PHB (>98% purity, Biomer, Germany) and an in–house PHBV standard (34% wt. HV content). The instrument was calibrated with pure zinc and indium standards according to Mettler-Toledo methods.

Polymer weight average molecular mass (in kDa) was estimated by solution rheology and intrinsic viscosity [η] measurements (dL/g). Polymer solutions (about 10 mL) were generated by combining weighed amounts of polymer dried film and DMC to result in known concentrations in the order of 15 mgPHA/mL. Polymer concentration was corrected for the film PHA purity that was estimated from the TGA measurements. Solutions were heated to dissolve the polymer in sealed glass digestion tubes (Hach, LZP065) for 5 min at 140°C, vortex mixed, then cooled and maintained in the heater block at 70°C. Viscosity of 5 mL aliquots of the heated solutions were measured at 60°C with a concentric cylinder measurement system rotating at a 75 s^{-1} shear rate (Anton-Paar MCR102 with a CC17 standard measuring system). Viscosity was estimated every 10 s from the average torque and over 5 min for each sample. Relative viscosities were estimated with respect to measurements of the solvent without added polymer and the intrinsic viscosity was calculated with the Solomon-Ciuta equation (Elias, 2008):

$$[\eta] = \frac{\sqrt{2\left(\eta_{sp} - ln(\eta_r)\right)}}{c} \quad (1)$$

where c is the PHA concentration (g/dL) in DMC, η_{sp} is the specific viscosity, and η_r is the relative viscosity. Validity of linearity assumptions for intrinsic viscosity estimated with this equation have been confirmed. Conversion of intrinsic viscosity to an estimated weight average molecular mass was made with the Mark-Houwink equation (Marchessault et al., 1970):

$$[\eta] = KM_w^{\alpha} \quad (2)$$

where K and α are empirical constants relating [η] (dL/g) to molecular weight (kDa). These Mark-Houwink constants, logK (-2.016 ± 0.025) and α (0.7384 ± 0.0098), were calibrated in separate research developments with respect to parallel samples dissolved in chloroform and molecular weight determined by gel permeation chromatography (GPC) with GPC calibration to polystyrene standards similarly to previously reported (Chan et al., 2017).

Polymer monomer composition was assessed by pyrolysis with gas chromatography and mass spectroscopy (PyGCMS). The evaluation gave the weight percent of 3-hydroxyvalerate (3HV) in the accumulated poly (3-hydroxybutyrate-co-3hydroxyvaleraye) (PHBV). A Gerstel (Mülheim an der Ruhr, Germany) pyrolysis unit was used with a Thermal Desorption Unit (TDU) and a liquid nitrogen Cooled Injection System (CIS). Grab samples of about 50 μg polymer particles, or polymer containing biomass particles, were deposited into an open ended clean 25 mm long (Gerstel 018131) quartz tube with a small retaining plug of quartz wool. Samples were loaded for pyrolysis by a Gerstel MPS 2XL autosampler. Pyrolysis was carried out under helium gas flow (60 mL/min) starting at 50°C for 0.5 min followed by heating at 120°C/min up to 710°C for pyrolysis unit, and 350°C for the TDU. The complete pyrolysis/TDU cycle lasted 8 min with maximum pyrolysis and TDU temperatures maintained. Pyrolysis products carried by the gas flow were trapped in the CIS at −100°C on a quartz wool filled liner in solvent vent mode. Gas chromatography (GC, Agilent Technologies 6890 N, Santa Clara, CA, USA) was employed using a Phenomenex ZB-5MS column (30 m, 0.25 mm diameter, 1 μm film thickness). The CIS and GC temperature programs were run in parallel enabling transfer and focus of analytes onto the head of the column with a split flow of 20 giving 1 mL/min constant helium flow in the column. CIS temperature program was from −100°C for 0.02 min, and then up to 280°C at 600°C/min. The GC oven temperature program was from 70°C for 5 min, and then up to 290°C at 10°C/min. The detector after GC was an Agilent Technologies 5975 XL Mass Selective Detector (MSD). MS data was acquired in scan mode with mass-to-charge (m/z) ratios ranging from 15 to 550 in a total scan time of 0.649 s. Pyrolysis, TDU and CIS were driven with Gerstel Maestro Controller (version 1.4.21.1) and MPS (version 1.4.15.1) drivers. GC and MSD were controlled by Evo3/Enhanced Masshunter GC/MS Acquisition (version B.07.06.2704). Acquired TIC (total ion chromatogram) data was processed and integrated using MassHunter Quantitative Analysis (Agilent Technologies, version B.07.00) while monitoring for the specific ion chromatogram (SIC) m/z 86 and 100. The SIC gave qualifying ions as markers for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate, respectively, since the pyrolysis process converts 3HB to 2-butenoic acid and 3HV to 2-pentenoic acid through a dehydration reaction (Khang et al., 2021). NIST MS Search (version 2.0, 2008) was used to confirm the identity of 2-butenoic acid and 2-pentenoic acid; match percentage exceeded 85% for both compounds. The integrated TIC peak ratio for 2-pentenoic acid with respect to the sum of 2-butenoic and 2-pentenoic acids was found to correlate directly to the 3HV wt% in PHBV with standards (Aldrich PHBV standards with 0, 5, 8 and 12% wt. 3HV content) and extracted PHBV using DMC. Correlation between SIC and TIC integrated peaks from pure standards was used to estimate the 3HV wt% for PHBV pyrolyzed in the more complex matrix of PHA-rich biomass samples. The SIC signal allowed to avoid interference from overlapping signal coming from non-PHA biomass.

2.6. Data analysis

The data collected during PHA accumulation tests were corrected for effects of sampling, acid addition for pH control and addition of feedstock (Johnson et al., 2009). The PHA content was expressed as mass fraction of the volatile suspended solids (gPHA/gVSS). The active biomass fraction or non-polymer biomass (X_a) was estimated as the total VSS minus PHA mass fraction. Active biomass was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (Johnson et al., 2009). The trend of change in PHA content was fitted by least squares regression to the empirical function:

$$\text{BiomassPHAcontent} = A_0 + A_1\left(1 - e^{-t/\tau}\right) \quad (3)$$

where A_0, A_1 and τ are constants that allow for estimation of rates as function of time (Bengtsson et al., 2017). Initial and average specific production/consumption rates and PHA yields on substrate were estimated for time 2 and 24 h, respectively. The average PHA yields on substrate were calculated on a COD-basis by dividing the amount of PHA (given 1.67 gCOD/gPHB) produced by the amount of substrate (given 1.07 gCOD/gHAc) added. Average specific production and consumption rates were calculated based on the cumulative amounts of acetic acid, PHA, biomass and oxygen consumed, with respect to estimated active biomass levels (gCOD/gX_a/h).

3. Results and discussion

Direct comparison between mixed culture PHA accumulation experiences is challenging, and even contradicting in outcomes that have been reported, due to underlying differences in approach, context and operations. In the present study, a consistent approach, context and method of operations were applied. The undertaking started with comparison in outcomes of polymer accumulation quantity between the

most commonly reported process conditions for PHA accumulation using full-scale municipal waste activated sludge as the PHA-storing biomass. First, continuous and pulse-wise feeding were evaluated. Pulse-wise feeding resulted in higher PHA contents and yields on substrate. Consequently, pulse-wise feeding was used as the platform for comparisons in further testing. Further testing included comparing process operating conditions such as pH control, the presence of active nitrification, and an influence of a biomass acclimation step prior to PHA accumulation. pH control and the presence of active nitrification were chosen because they can negatively affect the process performance due to an increase of salinity levels due to pH control and the reduction of available dissolved oxygen, due to the presence of active nitrification. Potential for influence of operations on the resultant polymer quality was characterized by solution rheology, thermogravimetric analysis, differential scanning calorimetry and pyrolysis–gas chromatography–mass spectrometry.

3.1. PHA production by municipal activated sludge

Municipal waste activated sludge can be reliably used as the source of biomass for the production of PHAs. In the present work, PHA accumulation tests were performed with activated sludge samples collected over a period of six months. An example of a PHA accumulation test with pulse-wise acetate feeding (F/M = 60 $mgCOD_{HAc}$/gVSS) is given in Fig. 1. Even though waste activated sludge was collected and used for PHA production at different time points, PHA contents and yields were observed to be consistently dependent on the process conditions used, as illustrated in Fig. 2. This experience replicates previous pilot scale outcomes. The same municipal activated sludge, as was used in this work, consistently produced commercial quality grades of PHA in 52 production batches spread over more than 10 months (Bengtsson et al., 2017). In the previous pilot study case, process conditions for PHA production were not changed during the experimental time and an average biomass PHA content of 0.41 gPHA/gVSS was obtained. Similar biomass PHA contents, 0.40 ± 0.01 gPHA/gVSS, were obtained in the present work under similar process conditions. These PHA contents are in line with expectations for PHA production with municipal activated sludge and are above the minimum PHA content required to make the process economically viable (Bengtsson et al., 2017).

Process conditions are known to influence PHA storage and biomass growth (Serafim et al., 2004). In all tests performed in this work, a preference for PHA storage over biomass growth was observed. In the first hours of the accumulation, ammonium accumulated in the reactor and biomass growth was not measurable, as illustrated in Fig. 1. By the end of the accumulation, low or negligible levels of ammonium, nitrite or nitrate were found in the reactor mixed liquor. Similarly, biomass respiration rates decreased in the first hours of the accumulation, but subsequently increased after 6-8 h with maximum respiration rates towards the end of the accumulation. An increase in biomass growth was observed after 6-8 h, as indicated by ammonium and oxygen consumption rates. This onset of active biomass growth can be explained by an adaptation of the biomass. It is likely that bacteria coming from conditions of famine would be in a state where the enzyme machinery necessary for cell growth would not be sufficiently induced, at least at the beginning of the accumulation test (van Aalst-van Leeuwen et al., 1997; Laycock et al. and Van Loosdrecht, 1999). PHA production acts as a buffer for the excess substrate that cannot be directed for biomass growth. In the present work, substrate uptake rates reached the maximum value directly in the first pulse, but growth rates only slowly increased. Consequently, more of the consumed acetate was channeled towards PHA storage at the beginning of the accumulation test. Gradually more acetate was directed to metabolism for biomass growth. This shift in metabolism is supported by observations of decrease in PHA yield on substrate, increase in ammonium removal, and increase in organic solids during the accumulation experiment (Fig. 1). A similar mechanism has been applied to explain the increase in growth rates observed after a pulse of substrate was given to an acetate limited chemostat culture (van Aalst-van Leeuwen et al., 1997). Research efforts should be directed to limit the growth response or on the contrary, to promote the growth of only the PHA-storing biomass fraction.

3.2. PHA accumulation performance under different feeding strategies

PHA contents and yields are consistently related to the applied feeding strategy. In general, higher PHA contents and average yields were obtained when the feedstock was supplied in pulses and not continuously (in excess). The feeding strategy with the lowest initial F/M ratio per pulse (20 $mgCOD_{HAc}$/gVSS) resulted in maximum PHA contents and average PHA yields on substrate, 0.34–0.35 gPHA/gVSS and 0.34–0.36 $gCOD_{PHA}/gCOD_{HAc}$, respectively. An overview of the observed PHA contents and yields on substrate can be found in Fig. 2 and 3. Under these low F/M pulse feeding, higher initial polymer yields on substrate were obtained and ranged from 0.53 to 0.57 $gCOD_{PHA}/gCOD_{HAc}$. Feeding in pulses with low COD concentration resulted in a high number of pulses, of up to 142 pulses given within 24 h. The effect of feedstock pulse-wise addition has been previously studied (Serafim et al., 2004; Serafim et al., 2008; Albuquerque et al., 2011; Chen et al., 2013; Valentino et al., 2015). For instance, Serafim et al. (2004) found higher PHA contents when the feedstock was added in three pulses instead of one. On the contrary, Albuquerque et al.

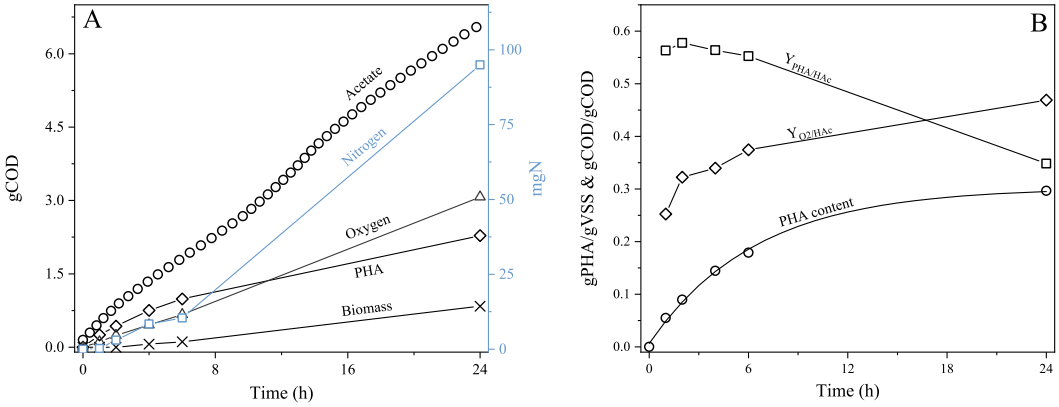

Fig. 1. Example of a PHA accumulation test with pulse-wise feeding with pulses reaching initial F/M ratios of 60 $mgCOD_{HAc}$/gVSS. A) Acetate, ammonium and oxygen are shown as cumulatively consumed while PHA and biomass are cumulatively produced. B) Yields on substrate are the average yields on substrate at selected times. Symbols are measured values and lines are included to help readability.

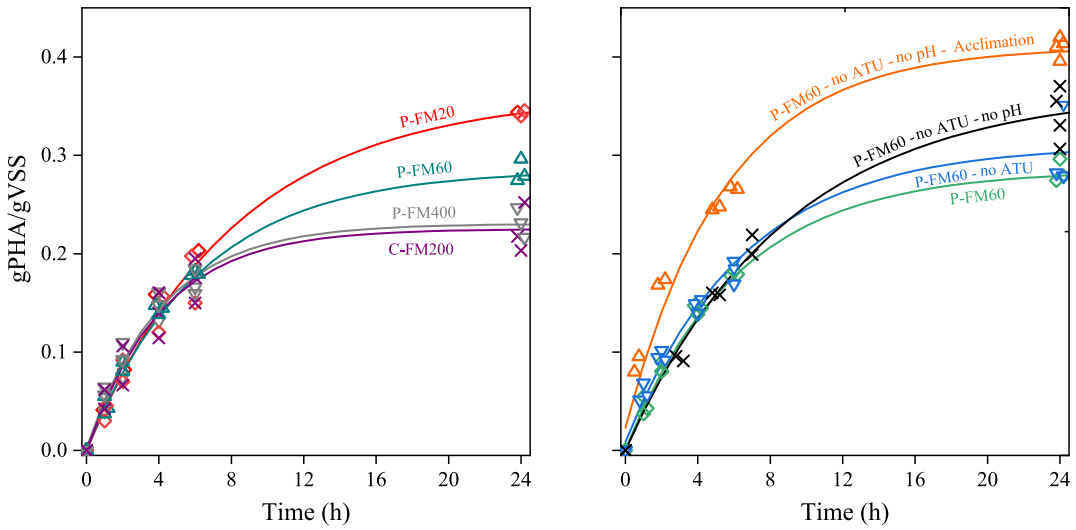

Fig. 2. Overview of the PHA contents obtained in all the PHA accumulation tests with continuous excess feeding (C), pulse-wise feeding (P), uncontrolled pH, active nitrification and an acclimation step.

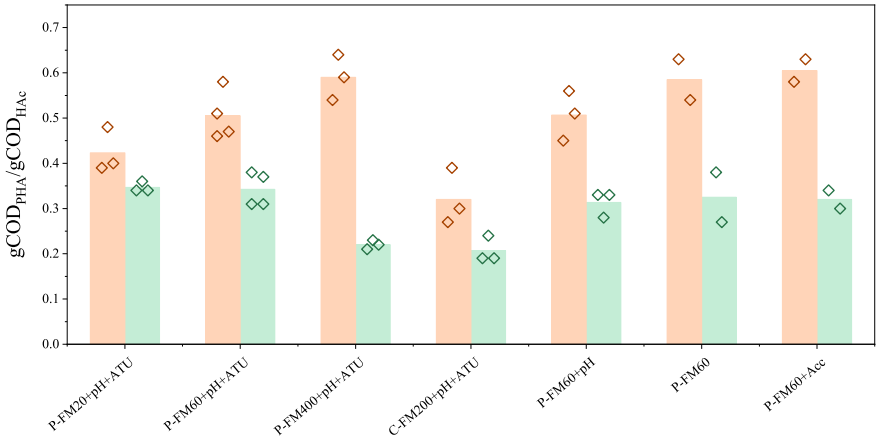

Fig. 3. Initial (orange) and average overall PHA yields on substrate (green) with continuous excess feeding (C) as a function of initial F/M ratio, given pulse-wise feeding (P), pH control (+pH), nitrification inhibition (+ATU), and acclimation (+Acc).

(2011) and Chen et al. (2013) found higher PHA contents when the feedstock was fed continuously compared to three or six pulses. Even though these studies compared pulse-wise to continuous excess feeding, the number of pulses was small (three to six pulses) compared to the pulse-feeding tested in the present work with up to 142 pulses.

As expected, the feeding strategy also affected the biomass production rates. Higher biomass production rates were observed in tests where the feedstock was dosed continuously or in pulses reaching 400 $mgCOD_{HAc}/gVSS$ (Fig. 3). When the substrate was added continuously or in pulses reaching 400 $mgCOD_{HAc}/gVSS$, the biomass production rate was 1.40–1.46 $gX/gVSS_0$, compared to those tests where the feedstock was dosed in pulses reaching peaks of 20 or 60 $mgCOD_{HAc}/gVSS$, where growth rates of 1.20–1.28 $gX/gVSS_0$ were observed. Even if the amount of biomass produced was higher, the average biomass yields on substrate were very similar between all the tested conditions. These trends were also observed in the ammonium removal rates, which were higher from the start of the test with continuous excess feeding or in pulses reaching 400 $mgCOD_{HAc}/gVSS$ (Fig. 5).

Differences in PHA contents, yields and biomass production under different initial F/M ratios have been previously explained in the context of substrate inhibition (Serafim et al., 2004). If this is the case, acetate and oxygen biomass uptake rates should be lower at higher substrate concentrations. In the present work, initial respiration rates were very similar among experiments and in the range 0.6–0.8 $mgO_2/(gVSS\ min)$, as observed in Fig. 4. No correlation could be found between a lower concentration of acetic acid per pulse and a higher initial respiration rate. For instance, in some tests with pulses reaching 400 $mgCOD_{HAc}/gVSS$, higher respiration rates were obtained compared to

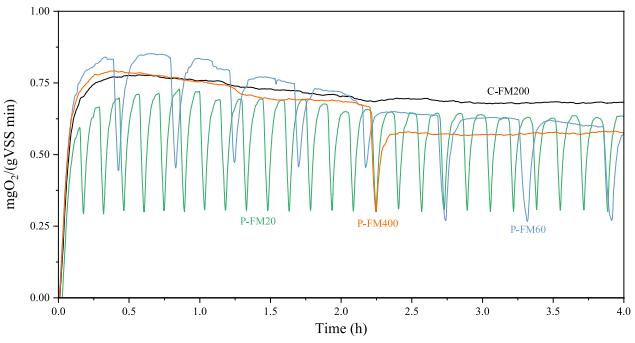

Fig. 4. Specific oxygen uptake rate in PHA accumulation tests with pulse-wise (P) and continuous excess feeding (C).

tests with pulses reaching 20 $mgCOD_{HAc}/gVSS$ and vice versa. Overall, oxygen consumption yields on substrate did not differ between tests with different feeding strategies, and they were in the range of 0.35–0.51 $gO_2/gCOD_{HAc}$.

The impact of dosing regime on the performance of accumulation requires careful consideration. The pulse-wise feeding strategy aims to still maintain the kinetics of polymer accumulation even though exogenous substrate supply begins to become exhausted before each subsequent pulse is triggered by a relative drop in respiration rate. A higher number of pulses will result in a higher number of interruptions in the availability exogenous of substrate. For instance, in the first six hours of a test with pulses reaching initial F/M ratios of 20 $mgCOD_{HAc}/gVSS$, 35 pulses were given. Between pulses, exogenous substrate was not available for an average of 105 ± 19 s. This pulse dead time resulted in almost 1 h out of the six hours with no exogenous substrate supply. If these dead times are too long and the endogenous substrate metabolic pools also become exhausted, then cells can stop their activity or even start to consume the produced PHA. If the next pulse is given "just-in-time" and the rate of exogenous mass transport of substrate into the cell is faster than the rate of polymer storage, then accumulation rates may be sustained despite the periodic (near) absences of exogenous substrate. On the contrary, when substrate was fed in excess by continuous feeding or in large pulses, interruptions of exogenous substrate supply are avoided. If the rate of polymer storage is not influenced by smaller or large pulse feeding then that would suggest that the inter-pulse dead time was sufficiently short to avoid repeated substrate limitation. Pulse feeding with periodic exogenous substrate limitation may result in a delay in biomass adaptation towards a growth response. The lack in such interruptions in the cases of excess (large pulse or excess continuous excess feeding) with sustained available exogenous substrate could explain why higher biomass growth and nitrogen removal rates were observed with excess feeding and few if any substrate interruptions, as observed in Fig. 5.

3.3. PHA accumulation performance without nitrification and/or pH control

Nitrogen compounds are known to influence growth and PHA production (Johnson et al., 2010; Fra-Vázquez et al., 2019). In addition, nitrifying bacteria, if present, would compete with the PHA-storing bacteria for ammonium, nitrite, and oxygen and can account for 25% of the overall oxygen consumption in activated sludge (Estévez-Alonso et al., 2021). As expected, in those accumulation tests where nitrification was not inhibited, an average of 20% higher oxygen consumption was measured. However, the PHA-storing bacteria themselves were not affected by the presence of active nitrifying bacteria. PHA contents and yields on substrate were similar to those obtained in control experiments with ATU addition and pH control, as observed in Fig. 2 and 3. This is likely due to the non-limiting dissolved oxygen concentrations. Even though nitrification was active, dissolved oxygen values were not lower than 1 mgO_2/L. Oxygen limitation is expected for concurrent PHA production and nitrification below this value (Estévez-Alonso et al., 2021). Co-existence of PHA-storing bacteria and active nitrification in enrichment reactors have been previously reported (Fra-Vázquez et al., 2019). Also in this case, active nitrification did not affect the enrichment of PHA-storing biomass. In industrial mixed culture PHA production, oxygen supply is expected to determine the maximum process volumetric productivity. Oxygen should preferentially be for PHA production and not for other metabolic processes in order to maximize volumetric productivities. Thus, nitrification needs to be minimized. For this purpose, several strategies that rely on the use of low dissolved oxygen concentrations (< 2 mgO_2/L) to limit the growth of nitrifying bacteria are available in literature (Estévez-Alonso et al., 2021). These lower dissolved oxygen concentrations have a more negative effect on nitrifying than in PHA-storing bacteria.

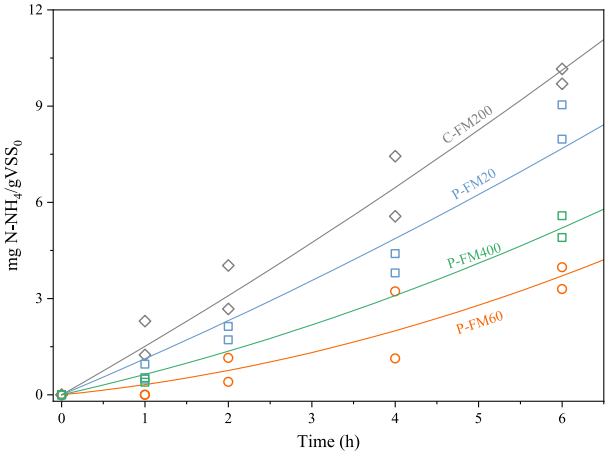

Fig. 5. Differences observed in nitrogen consumption per gram of initial biomass in the first six hours of the PHA accumulation tests with continuous and pulse-wise feeding with a pulses reaching initial F/M ratios of 60 $mgCOD_{HAc}/gVSS$.

A high salinity level can have a negative influence on bacterial growth and PHA production (Palmeiro-Sánchez et al., 2016). In the present work, the feedstock addition and the use of pH control resulted in the addition of 3.1–4.2 gK+ and 3.5–4.7 gCl-. To reduce the concentration buildup of salts in the reactor, experiments without pH control were performed. The obtained pH profile was different from previous experiments. Initial pH values ranged between 7.5 to 8.0, but when pH was not controlled, it slowly increased towards pH 9.0. Despite a lower total amount of salts added to the reactor when pH was not controlled, results of PHA content and yields on substrate were similar, as observed in Fig. 2 and 3. These results do not support what has been previously reported (Liu et al., 2011; Serafim et al., 2004). Higher PHA contents and yields have been reported when pH was not controlled or was controlled at pH values between 8 and 9. When pH was not controlled, initial pH was between 7 and 9 and final pH ranged from 8 to 9. Nonetheless, the highest PHA content ever reported with mixed cultures was obtained when pH was controlled to a value of 7 ± 0.1 (Johnson et al., 2009). From the results obtained in this work, it can be concluded that at industrial PHA production with waste activated sludge, the use of pH control can probably be avoided, and consequently process costs due to amounts of chemicals added for pH control can be avoided.

3.4. PHA accumulation performance with a biomass acclimation step

A biomass acclimation step prior to the PHA accumulation has been shown to promote higher accumulation degrees in PHA production by municipal activated sludge (Morgan-Sagastume et al., 2017; Bengtsson et al., 2017). In the present work, higher PHA contents were obtained when the biomass was subjected to three short feast and famine cycles. PHA contents in tests with biomass acclimation were 0.40–0.42 gPHA/gVSS compared to only 0.28–0.37 to tests without an acclimation step. Initial PHA production rates were also higher when an acclimation step was applied, as shown in Fig. 2. Despite the fact that higher acetate uptake rates and PHA contents were observed, average PHA yields on substrate did not change compared to control experiments. These yields were in the range of 0.30–0.34 $gCOD_{PHA}/gCOD_{HAc}$. Higher yields on substrate were obtained in previous studies (Morgan-Sagastume et al., 2017). The reason why similarly high yields were not obtained in this work remains unclear. A fundamental understanding of acclimation adaptation was not part of the current work and further research is required. The proposed mechanism(s) behind this adaptation response could be related to a stimulation for growth of the PHA-storing bacteria, the waking-up of a dormant biomass fraction present in the activated

sludge, or a physiological adaptation of the PHA-storing cells. Based on previous research, this acclimation step is understood to be a physiological adaptation or an activation of dormant cells rather than the growth of the storing biomass. An increase in the final PHA content from 0.3 to 0.4 gPHA/gVSS cannot be explained only by an increase in PHA-storing biomass. If it is assumed that the PHA-storing bacteria can accumulate a maximum of 0.6 gPHA/gVSS (Pei et al., 2022) and the initial VSS concentration in the reactor is 2.5 gVSS, an increase in the final PHA content from 0.3 to 0.4 gPHA/gVSS, would mean that 25% more PHA-storing biomass (625 mgVSS) should have been formed in the acclimation step compared to experiments when the acclimation step was not performed. This level of biomass increase is not possible because the amount of COD added in these three cycles, only 450 mgCOD. These amounts of COD could result in the production of 165 mgVSS.. Ongoing investigation is directed to elucidate the mechanism(s) behind the acclimation response.

3.5. Polymer recovery and recovered polymer properties

Polymer properties, in the biomass and for extracted films, were evaluated for triplicate batches of the harvested, acid-washed, and dried biomass from 5 of the accumulation tests (see supplementary material). The five batches comprised four sets made with pH control and ATU addition (P-FM20 + pH + ATU, P-FM60 + pH + ATU, P-FM400 + pH + ATU, and continuous excess feed), and one set with pH control, but without ATU addition (P-FM60 + pH). The average 3HV content measured for produced PHBV in the biomass was low (1 ± 1% wt., n = 15). Negligible relative amount of 3HV was expected given acetic acid as sole substrate. Ash (inorganic content) of the dried biomass solids was high (21 ± 3% g/g, n = 15) and this contributed to a significant total biomass mass loading for targeted polymer extraction concentrations of 20 mgPHA/mL. Extraction biomass loading was also high due to lower biomass PHA contents on average (16 to 31 gPHA/gTS), because these accumulations were made without the acclimation step. However, extractions could still be readily made. Following an assessment of polymer properties and extraction yields, statistical evidence for any effect of polymer impurity level on assessed properties, polymer properties on extraction outcomes, and accumulation conditions on polymer properties was tested.

A consistent level of 4.1 ± 0.6% wt. of the non-polymer biomass was co-extracted. Therefore, differences in biomass mass loadings for the targeted 20 mgPHA/L polymer extraction concentration resulted in a corresponding range in variability of the total extract purity from 79 to 89% PHA. The extractable amount of PHBV was also found to be variable and ranged from 61 to 100%. The polymer melt behaviour was assessed directly in the dried biomass and for the recovered polymer film based on the second melting ramp. Melt peak enthalpy and temperature for the PHA-in biomass, and PHA-film, were 63 ± 9 J/gPHA and 175 ± 1°C, and 91 ± 9 J/gPHA and 173 ± 1 °C. For reference, the same peak enthalpy and melt temperature for Biomer PHB was 102 J/gPHA and 175°C (M_w = 1325 kDa). Melt peak temperatures were similar in all cases, but melt enthalpies were lower than the commercial PHB for the recovered films, and significantly lower for the PHA-in biomass. A minor component of 3HV in the PHBV produced could lower the expected melt enthalpy compared to pure PHB. Acid-washing of the biomass before drying promoted a consistently high thermal decomposition temperature (T_d = 287 ± 2°C at 10°C/min, n = 15) as was expected (Chan et al., 2017). A high thermal decomposition temperature is essential for mitigating polymer molecular weight loss for high temperature drying and solvent extraction (Werker et al., 2013a; Werker et al., 2013b; Werker et al., 2020; Bengtsson et al., 2017). The extracted PHBV weight average molecular mass was consistently high on average but still variable from the 15 accumulation batches in the range from 1000 to 2000 kDa, as observed in Fig. 6. This range of PHBV molecular weight is uniquely higher on average than typically reported for mixed culture PHA production (Serafim et al., 2008; Bengtsson et al., 2010; Albuquerque et al., 2011; Palmeiro-Sánchez et al., 2016; Lorini et al., 2021; Rodrigues et al., 2022). Higher molecular weights have been shown to result if the accumulated PHA-rich biomass, after acid-washing, is dried by first heated rapidly from ambient conditions (Werker et al., 2020; Werker et al., 2013b). An increase in heating rate was achieved by disposing the biomass to a higher initial oven temperature (120°C for 30 min and then drying at 105°C overnight).

There was no correlation to suggest that the different relative levels of co-extracted non-polymer biomass, that influenced extracted polymer purity, affected measured melt properties or molecular weights by solution rheology. Interestingly, the concentration of dissolved non-polymer biomass in the solvent after extraction was correlated to both the concentration of extracted polymer (P = 0.0112) as well as the mass of non-polymer biomass loaded into the tube for the extraction (P < 0.0001). Multi-linear regression suggested that 1 mg/gDMC of non-polymer biomass used in the extraction would result in 0.03 mg/

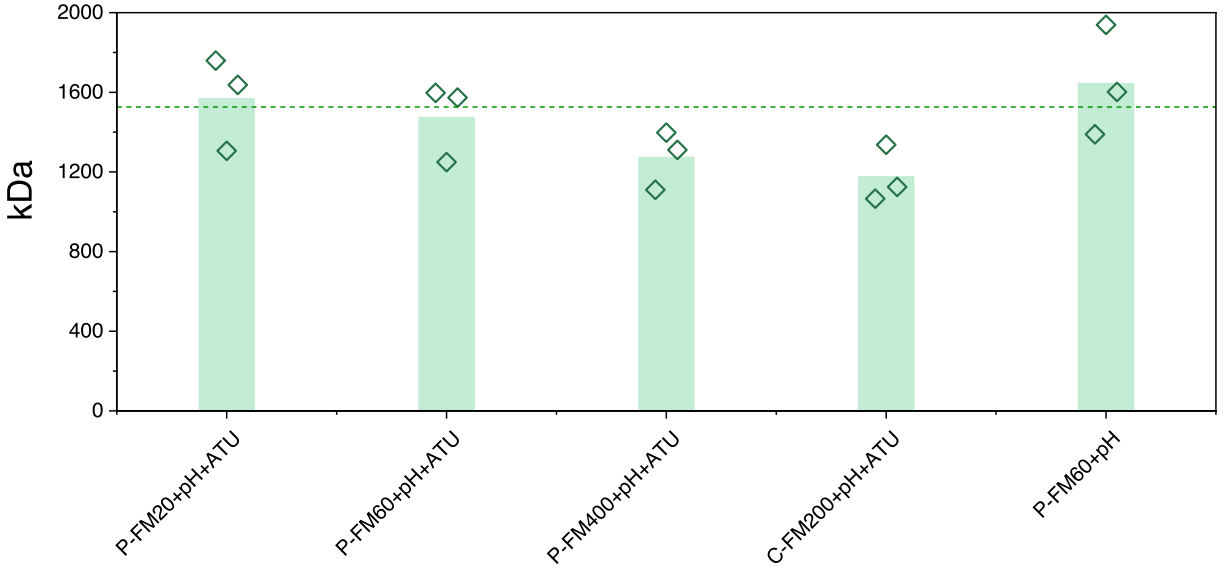

Fig. 6. Overview of PHA molecular weights obtained at the end of the PHA accumulation tests with continuous excess feeding and pulse-wise feeding and subsequent standardized polymer extraction. Dash line indicates the molecular weight average value for the pulse feeding without excess substrate (P-FM20 and P-FM60).

gDMC dissolved biomass impurity. Additionally, every mgPHA/gDMC extracted polymer carried an estimated amount of 0.07 mg/gDMC impurity into solution in this particular study. Even if co-extracted non-polymer impurities can be removed after extraction, the challenges and costs to reach a polymer product with high purity (> 98%) would be reduced if the polymer content in the biomass is higher.

Extraction recoveries were lower than expected based on previous experience with similar dried biomass having higher biomass PHBV contents. The concentration of PHBV that became dissolved in the DMC was naturally a function of how much biomass containing PHBV was loaded to the extraction system ($P < 0.0001$). It was predicted that on average for every mgPHA/gDMC loaded to the extraction tube, 0.8 mgPHA/gDMC became dissolved in solution. However, it was also found that the extraction recovery was correlated to the expressed level of melt (or crystallization) enthalpy ($P = 0.0002$). The melt enthalpy of the PHA in the biomass was on average 70% of the level for the same polymer extracted from the biomass. Bacteria store PHA as granules (so-called carbonosomes (Jendrossek and Pfeiffer, 2014)), and the polymers within in vivo granules are maintained in a hydrated non-crystalline state enabling metabolic activity for polymer storage and its mobilization. These observed in situ melt property dependent extraction recoveries suggest that the more the polymer in the biomass expresses thermoplastic behaviour as a polymer, the more it can be readily extracted. Therefore, methods of downstream processing before solvent extraction should work to maximize that the PHA coalesces (Sedlacek et al., 2019) to behave more as a thermoplastic polymer and less like its native carbonosomes state in vivo.

Extraction recovery was also negatively correlated to the recovered polymer molecular weight ($P = 0.0014$). The negative influence of molecular weight on extraction recovery introduces a practical consideration for commercial developments. Diffusion of solvent, dissolving the polymer, and polymer transport into solution are likely to be negatively influenced by increased solution viscosity and a greater tendency for gel formation that could block the biomass particle pore spaces. Higher molecular weight polymers may offer unique opportunities for certain kinds of applications (Bengtsson et al., 2017). However, higher molecular weight can, according to these results, reduce extraction performance. That decease would decrease commercial productivity at an industrial scale. Engineering to modulate molecular weight during recovery to be as high (or low) as absolutely required for the polymer intended application is a necessary aspect for balance in value from quality and quantity at industrial scale. The standardized extraction methods applied in the present work can be adapted and applied for assessments for tuning industrial scale conditions.

The observed molecular weight variability could have been influenced by effects of unforeseen/uncontrolled differences during drying and extraction, and/or in the accumulation operations. The accumulation results were grouped between pulse feeding methods (P-FM20, and P-FM60 feeding), and excess feeding methods (P-FM400, continuous excess feeding). Excess feeding methods resulted in statistically significantly lower yield of PHA on substrate, and lower weight average molecular mass. This correspondence suggests that better yield on substrate promotes conditions for generating polymers with higher molecular weight. It is considered that if the bacteria divert less carbon to polymer storage (excess feeding method outcomes), then the carbon flux for polymer formation will not be at its maximum possible rate. Loss of flux for rate limitation in polymer formation kinetics has been speculated to increase the inherent chain termination probability for polymerization (Werker et al., 2013a). Increase in the chain termination probability results in polymer chains with lower average molecular weight. Indications that lower yields lead to lower polymer molecular weights may nevertheless be coincidental and so these insights require continued systematic evaluations. Neglecting the excess feeding method outcomes, the molecular weight for the pulse feeding results was 1536 ± 221 kDa. This level sets a higher expected standard for what mixed culture PHA production should be able to consistently achieve. At the same time, the underlying causes for the unexplained variability of ± 221 kDa in molecular weight outcomes remains a challenge to understand further in the ongoing developments.

4. Conclusions

Waste activated sludge can be directly used for PHA production. An acclimation step combined with pulse-wise feeding strategy resulted in maximum PHA contents of 0.40–0.42 gPHA/gVSS and product yields of 0.30–0.34 $gCOD_{PHA}/gCOD_{HAc}$. Nitrification and pH control did not have an observable effect on the PHA production capacity of the biomass. PHAs with molecular weights in the order of 1500 kDa were obtained. Polymer molecular weight and thermoplastic properties in the dried biomass influenced polymer extraction recovery. A standard protocol for an activated sludge PHA accumulation test, including downstream processing and extraction, has been made available as supplementary material.

CRediT authorship contribution statement

Ángel Estévez-Alonso: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Beatriz Altamira-Algarra:** Investigation, Writing - review & editing. **César Arnau-Segarra:** Investigation, Writing - review & editing. **Mark C.M. van Loosdrecht:** Conceptualization, Supervision, Writing - review & editing. **Robbert Kleerebezem:** Conceptualization, Supervision, Writing - review & editing. **Alan Werker:** Conceptualization, Investigation, Formal analysis, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, athttps://doi.org/10.1016/j.biortech.2022.128035.

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