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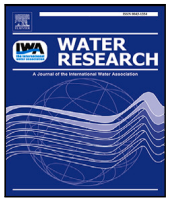

Influence of environmental conditions on accumulated polyhydroxybutyrate in municipal activated sludge

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ABSTRACT

Poly(3-hydroxybutyrate) (PHB) was accumulated in full-scale municipal waste activated sludge at pilot scale. After accumulation, the fate of the PHB-rich biomass was evaluated over two weeks as a function of initial pH (5.5, 7.0 and 10), and incubation temperature (25, 37 and 55 °C), with or without aeration. PHB became consumed under aerobic conditions as expected with first order rate constants in the range of 0.19 to 0.55 d^{-1}. Under anaerobic conditions, up to 63 percent of the PHB became consumed within the first day (initial pH 7, 55 °C). Subsequently, with continued anaerobic conditions, the polymer content remained stable in the biomass. Degradation rates were lower for acidic anaerobic incubation conditions at a lower temperature (25 °C). Polymer thermal properties were measured in the dried PHB-rich biomass and for the polymer recovered by solvent extraction using dimethyl carbonate. PHB quality changes in dried biomass, indicated by differences in polymer melt enthalpy, correlated to differences in the extent of PHB extractability. Differences in the expressed PHB-in-biomass melt enthalpy that correlated to the polymer extractability suggested that yields of polymer recovery by extraction can be influenced by the state or quality of the polymer generated during downstream processing. Different post-accumulation process biomass management environments were found to influence the polymer quality and can also influence the extraction of non-polymer biomass. An acidic post-accumulation environment resulted in higher melt enthalpies in the biomass and, consequently, higher extraction efficiencies. Overall, acidic environmental conditions were found to be favourable for preserving both quantity and quality after PHB accumulation in activated sludge.

1. Introduction

Polyhydroxyalkanoates (PHAs) are a family of naturally occurring polyesters that accumulate intracellularly in many species of microorganisms as an endogenous organic substrate (Dawes and Senior, 1973). These microorganisms accumulate PHA when they are subject to dynamic environments when, for example, surplus organic carbon sources become suddenly available while other growth factors, such as nitrogen and phosphorus, are limiting (Van Loosdrecht et al., 1997; Majone et al., 1999; Reis et al., 2003). PHAs can be accumulated in microbial biomass within engineered bioprocesses to significant levels (0 40 to 0 90 grams PHA per gram organic mass) (Estévez-Alonso et al., 2021). Accumulated intracellular PHA can be extracted and purified. Purified PHAs exhibit thermoplastic properties similar to fossil derived polyesters (Raza et al., 2018). Compared to fossil-based plastics, plastics made from PHA are bio-based and can be completely biodegradable. Thus, PHAs are an anticipated renewable resource for use in applications, especially where biodegradation is required.

PHAs are produced commercially today with pure culture methods, using refined substrates and sterilisation. PHA plastics compete with a higher selling price compared to conventional petroleum derived plastics, and this limits PHA market penetration (Plastics Europe, 2020; Vandi et al., 2018). Cost reduction has been an underlying motivation driving much fundamental research and development in methods and processes for PHA production. Over the past two decades, much advancement has been made in the use of open culture production methods and waste organic residuals for substrates as a principal strategy to reduce PHA production costs (Estévez-Alonso et al., 2021; Reis et al., 2003).

Municipal biological wastewater treatment processes produce significant amounts of waste activated sludge. Municipal activated sludge can exhibit a significant degree of enrichment for the PHA-storing phenotype and can be directly used for PHA accumulation. Pei et al. (2022c) and Estévez-Alonso et al. (2021). PHA-accumulating bacteria can exist in the waste activated sludge due to the dynamics in the

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process environments or dynamics in daily loading conditions, which make metabolism involving polymer storage inherently a good selective strategy for survival (Van Loosdrecht et al., 1997). In recent years, technical viability for using waste activated sludge for PHA production has progressed from lab to pilot scale (Estévez-Alonso et al., 2021; Bengtsson et al., 2017; Valentino et al., 2018). Pilot scale production trials have exhibited stable outcomes in quantity and quality of polymers produced with average biomass PHA content with respect to volatile solids (VS) of 0.4 gPHA/gVS (Estévez-Alonso et al., 2021; Reis et al., 2003). More recent investigations suggest expectations for optimal waste activated sludge accumulation to reach about 0.64 gPHA/gVS which is similar to typical expectations in general for both pure and open culture production methods alike (Pei et al., 2022a).

The direct use of activated sludge for PHA production has two main process components: PHA accumulation and downstream processing (DSP). PHA accumulation is an aerobic bioprocess where the activated sludge is fed with a volatile fatty acid (VFA) rich substrate to reach the maximum possible biomass PHA content. DSP starts after PHA accumulation with steps to separate process water and recover a purified PHA from the biomass suspended solids. DSP methods are less well understood for the up-scaling of open culture PHA production and are a current knowledge gap needed for industrial-scale developments (Estévez-Alonso et al., 2021). DSP steps are critical to preserving polymer quantity and quality within the harvested post-accumulation PHA-rich biomass and contribute heavily to the overall process economics (Werker et al., 2020; Kourmentza et al., 2017).

Published research developments for DSP are commonly focused on specific purification steps. Towards this aim, comparisons are made for different approaches or conditions for smaller subsamples from a batch of PHA-rich biomass preserved directly after accumulation by laboratory scale centrifugation, followed by thermal drying or lyophilisation. However, the fate of the PHA directly after accumulation and the stability of the freshly made product has not been systematically studied.

Microorganisms metabolise PHA not only during starvation but also to mitigate stresses (Müller-Santos et al., 2021). Changes in environmental conditions (i.e. osmotic pressure, oxygen level, pH and temperature) after PHA accumulation can also promote PHA mass loss and property changes. Abiotic random scission due to β elimination in the presence of H^+ or OH^- will reduce molecular weight (Lauzier et al., 1994). Intracellular and extracellular PHA depolymerases are anticipated to still be active directly after the PHA accumulation process. Intracellular PHA depolymerases that are integral to the surface of the PHA granules (Jendrossek, 2009). They are reported to act on intracellular PHA in its so-called native amorphous hydrated state (Jendrossek, 2009; Oeding and Schlegel, 1973; Ong et al., 2017). Meanwhile, extracellular depolymerases are understood to degrade PHA from denatured granules from lysed cells, and for this semi-crystalline polymer in nature (Ong et al., 2017; Jendrossek and Handrick, 2002). Directly after the PHA accumulation process, in the absence of any cell lysis, PHA will be stored intracellularly in host cells as bioactive functioning granules. Therefore, factors and environments that can influence the level of intracellular PHA depolymerase activity should, at least initially, determine the polymer fate.

The metabolism of intracellular PHA degradation is still not completely clear. For poly(3-hydroxybutyrate) (PHB), the depolymerase enzyme hydrolysis leads to 3-hydroxybutyrate or 3-hydroxybutyryl coenzyme A and then enters the β-oxidation cycle to be further metabolised (Eggers and Steinbüchel, 2013; Uchino et al., 2007; Gebauer and Jendrossek, 2006; Jendrossek and Handrick, 2002). The degradation process may require the activation of released 3-hydroxybutyrate to 3-hydroxybutyryl coenzyme A which is an energy-consuming reaction (Eggers and Steinbüchel, 2013). The intracellular depolymerase activity level can be influenced by environmental conditions and/or polymer properties. Environmental conditions such as temperature, pH, and oxygen concentration can affect enzyme production, activity, and stability. Due to enzyme substrate specificity, research on intracellular depolymerase properties requires native PHA granules, which can be challenging to isolate (Jendrossek and Handrick, 2002). Hence, it is not straightforward to systematically evaluate optimum temperatures or pH levels for all possible and potentially different intracellular depolymerase activities.

During the initial steps of DSP, material management methods can include a need in the logistics to stage the freshly produced PHA-rich biomass with temporary storage. Handling and time scales of the industrial processes before the final DSP steps of purification can result in PHA mass and property losses. For example, even heating rates in biomass thermal drying significantly influence product molecular weight (Estévez-Alonso et al., 2022). The risks for polymer loss and property changes directly after the PHA accumulation may be expected to be negligible in gram laboratory- and even kilogramme pilot scale PHA production research and development work. Due to the relatively low volumes and masses of material to be handled, the steps of DSP can be managed quickly (minutes to a few hours). At the industrial scale, with significantly higher expected process volumes and masses of material to handle, duty cycles for each fresh batch will be longer (hours to days). PHA-rich biomass can be produced at a number of distributed sites, and at least some DSP steps, including final drying, can be handled more centrally with improved scale of economy as done today for municipal sludge management. Therefore, the transport of dewatered PHA-rich biomass may benefit the production economics. Quicker handling requires higher throughput (larger) equipment and associated capital costs. Smaller equipment would require that PHA-rich biomass be temporarily stored in DSP feed tanks.

The stability of the PHA in the fresh crude product of accumulated PHA-rich biomass has not been reported in the research literature. The effects of time and environmental conditions in the storage of PHA-rich biomass fate are overlooked. Therefore, it was of interest to explicitly evaluate how well freshly accumulated PHA survived this stage with environmental conditions from acidic to basic pH, mesophilic to thermophilic temperatures, and with or without the presence of oxygen. For the present investigation, the effect of environmental conditions (pH, temperature, aeration) on the fate of freshly accumulated PHB in municipal activated sludge was evaluated. A pilot scale process was operated with an acetic acid feedstock to yield a PHB-rich biomass. The harvested PHB-rich biomass mixed liquor was divided into parallel batches. Parallel batches were pH adjusted and incubated at selected temperatures, with and without aeration. Sampling and monitoring of polymer fate over time for mass balances and property evaluations were undertaken and interpreted as reported herein.

2. Materials and methods

2.1. PHB accumulation

Waste activated sludge was obtained from Bath Wastewater Treatment Plant (WWTP, Rilland–Bath, the Netherlands). This WWTP treats a mixture of municipal and industrial influent wastewater for 470,000 person equivalents. The WWTP includes primary treatment followed by a modified Ludzack–Ettinger activated sludge biological process. Phosphorus is removed by $FeCl_3$ precipitation. Batches of fresh grab samples of gravity belt thickened WAS (56.7 gTS/L) were taken and delivered on September 5th, November 4th and December 3rd, 2019. The thickened waste activated sludge was stored at 5 °C pending its use in accumulation experiments. Accumulation production experiments were performed with respective batches on September 25th, November 5th and December 4th, 2019. The freshly accumulated PHB-rich biomass was the starting material used for the principal incubation experiments. Control incubation experiments were also performed with the same activated sludge but without any polymer accumulation.

The feedstock for PHB-rich biomass production was 20 gCOD / L acetic acid (VWR, the Netherlands) with added NH_4Cl and KH_2PO_4

(VWR, the Netherlands) for a COD:N:P (by weight) of 100:1:0.05. The pH was adjusted to 5.0 ± 0.5 with NaOH (VWR, the Netherlands). The use of acetic acid ensured that the produced polymer was PHB (Lemos et al., 2006).

The pilot scale accumulation process was carried out in a jacketed stainless steel 200 L reactor with 167 L working volume as described in Pei et al. (2022a) (Figure S1). Accumulation experiments were initiated by loading the reactor with a set mass of the thickened waste activated sludge that was diluted with tap water to a starting suspended solids concentration of about 4 gVSS / L. The reactor was operated at 25 °C with constant mixing (230 rpm) and aeration (50 L/min). Before the accumulation process started, a period of steady aeration was applied for about 12 h hours to reach a steady level of endogenous respiration. This steady level was determined from respiration levels based on the dissolved oxygen trends (Pei et al., 2022a).

The accumulation process was fed-batch with a constant process volume and constant aeration. Aeration maintained dissolved oxygen levels over 1 mgO_2/L. Reactor volume continuous circulation through a clarifier (16 L) maintained a constant overall process volume with retention of the suspended solids. The substrate was pumped in discrete pulses of a fixed volume to target peak pulse substrate concentrations of 100 mgCOD / L. Pulse input timing was from feedback control that was triggered by respiration level monitoring based on dissolved oxygen trend measurements (JUMO ecoLine O-DO, JUMO GmbH & Co. KG, Germany) as previously described by Werker et al. (2020). The process started with an acclimation step (Morgan-Sagastume et al., 2017) using three acclimation pulses as previously reported (Pei et al., 2022b). After the third acclimation cycle, the accumulation process was directly started. During accumulation, feed pulses were given intact with the substrate uptake rate based on dissolved oxygen trends and the tight series of input pulses were thereby given without excess substrate supply and build up in the reactor over 20 h.

2.2. PHB-rich biomass incubation experiments

Three replicate batches of PHB-rich biomass were produced and used directly after 20 h accumulation for the purposes of the incubation experiments. Accumulation was terminated by switching off aeration, but mechanical mixing was sustained to maintain a well-mixed liquor. After accumulations, pH was around 8, and since a final pulse of the substrate was given before aeration was terminated, the initial background acetic acid concentration was about 100 mgCOD/L.

For each incubation experiment, three 10 L grab samples of mixed liquor were taken from the accumulation reactor. The initial pH of the grab samples was adjusted to pH 5 5, 7 and 10 using 1M HCl or 1M NaOH, respectively. During the incubation experiment, pH was thereafter monitored but not further controlled.

Respective batches of pH adjusted mixed liquor were then distributed in replicate 170 mL aliquots to 21 × 250 mL stoppered serum bottles and 9 × 500 mL Erlenmeyer flasks with silicone sponge stopper. Serum bottle headspace was exchanged with nitrogen gas with overpressure to establish and maintain anaerobic incubation conditions. A Discofix® one-way Stopcock (B. Braun, Germany) together with a 40 mm needle was connected to each of the anaerobic serum bottles. This connection had a radially and axially movable swivel lock for a safe and quick connection, and it was used for liquid/gas sampling. Erlenmeyer flasks with porous silicone sponge stoppers were used for aerobic incubations. Serum bottles and Erlenmeyer flasks were incubated on an orbital shaker table at 120 rpm and at a constant selected temperature (25, 37 or 55 °C).

The incubation period was for up to 15 days. During weekdays, daily grab samples were taken in triplicate using 3 of the incubation vessels for each condition defined by initial pH, incubation temperature, and with or without aeration. The remaining unsampled serum bottles provided a mass of material for end-point assessment with the characterisation of the resulting suspended solids and polymer quality.

2.3. Control incubation experiments

Control experiment 1 was conducted to evaluate the fate of exogenous PHB due to extracellular depolymerase activity as a function of initial pH. Three 1.3 L jacketed batch reactors were operated isothermally at 37 °C in batch mode under selected constant pH (5 5, 7 and 10). pH was monitored and controlled by dosing inorganic acid/base. To ensure anaerobic conditions, the reactors were flushed with N_2 gas. An aliquot of nominally 200 g waste activated sludge containing negligible PHA also from Bath WWTP was incubated with 2.5 g commercial PHB (>98% purity, Biomer Germany) with the particle size of 273±13 μm. In addition, about 30 g of inoculum from a full-scale anaerobic digestor was used to ensure the presence of active acidogenic activity. Two grams 2-bromoethanesulfonate were used to inhibit methanogenesis activity.

Control experiment 2 was carried out to evaluate the PHB hydrolysis. Pure (>98 wt %) commercial PHB powder (Biomer, Germany) was incubated at a concentration of 1 g/L in triplicate 100 mL with closed serum bottles. The initial pH in the serum bottles were adjusted to 5 5, 7 or 10. The serum bottles were incubated at 120 rpm and 37 °C. 3-Hydroxybutyrate and VFA concentrations were monitored over time.

Control experiment 3 was conducted to evaluate the generic enzyme activity towards PHB. Pure (>98 wt%) commercial PHB powder (Biomer, Germany) at a concentration of 1 g/L was combined with lipase enzyme Lipozyme® CALB (Novozymes, Denmark) from *Candida antartica* B at a concentration of 10 mL/L in triplicate 100 mL closed serum bottles. The serum bottles were incubated at 120 rpm and 37 °C. 3-Hydroxybutyrate and VFA concentrations were monitored over time.

2.4. Analytical methods

Figure S2 shows the sample analysis workflow schematically. The mixed liquor pH (SevenExcellence S400 Basic, Mettler Toledo, Switzerland) and total Chemical Oxygen Demand (tCOD) were monitored. tCOD samples were diluted up to 10 times with Milli-Q water (Merck, Germany) for cuvette COD test kits (Hach Lange, USA).

Headspace samples were taken from the experimental stoppered serum bottles by syringe. Biogas formation was detected from gas chromatography (Varian CP-4900 Micro-GC, Varian, UK). The gas chromatograph was equipped with Mol Sieve 5 ÅPLOT (MS5) (10 m × 0.53 mm, 30 μm, fused silica, aluminosilicate phase) column and a thermal conductivity detector.

For soluble water quality analyses, mixed liquor samples were centrifuged (3248 RCF for 20 min at 4 °C, Beckman Coulter, CA, USA). The pellet was saved for suspended solids quality analyses, and the decanted supernatant was filtered (1 μm GF/0.45 μm PVDF Phenex ™-GF/PVDF Syringe Filters, Phenomenex, USA).

The filtered liquid samples were assessed for soluble Chemical Oxygen Demand (sCOD), VFAs, hydroxybutyric acid, anion, and cation concentrations. sCOD was determined with the COD cuvette test kits depending on the concentration (Hach Lange, USA). VFAs and hydroxybutyric acid concentrations were measured by Dionex ultra-high-pressure liquid chromatography system with Phenomenex Rezex organic acid H^+ column (300 × 7.8 mm) and a Dionex Ultimate 3000 RS UV detector (210 nm) with 2.5 mM sulphuric acid mobile phase at a flow rate of 0.5 mL/min and 80 °C. Anions were determined by ion chromatography (Metrohm 761 Compact IC, Metrohm, Switzerland) with a built-in conductivity detector. A pre-column (Metrohm Metrosep A Supp 4/5 Guard) and a column (Metrohm Metrosep A Supp 5, 150/4.0 mm) were used with 3.2 mM sodium carbonate and 1 mM sodium hydrogen carbonate + 1% acetone solution mobile phase at 0.7 mL/mL at room temperature. A chemical suppressor was also applied (0.2 M phosphoric acid + 1% acetone at 0.1 mL/mL). Ion chromatography was also used to assess cation concentrations (Metrohm Compact IC Flex 930 Metrohm, Switzerland). The ion chromatograph was equipped with a pre-column (Metrohm Metrosep RP 2 Guard/3.6), a column (Metrohm

Metrosep C 4–150/4.0 mm) and a conductivity detector. HNO_3 with a concentration of 3 mM was used as a mobile phase at a flow rate of 0.9 mL/min.

The collected suspended solids pellet of PHB-rich biomass, after the centrifugation, was first re-suspended with Milli-Q water (Merck, Germany) and then acidified down to pH 2 by addition of 98% H_2SO_4 (VWR, the Netherlands). The acidified sample was mixed (5–10 min), centrifuged (3248 RCF for 5 min at 4 °C), and the supernatant was removed. The acidified pellet was again re-suspended with Milli-Q water to remove residual H_2SO_4, centrifuged as before, to re-collect the now acidified and washed pellet. The pellet was dried at 105 °C overnight (12 h), weighed, and then ground to a fine powder. These dried and ground biomass solids were characterised by TGA and DSC (see below).

Carbon, nitrogen, hydrogen, and oxygen composition for the PHB-rich biomass was measured by elemental analysis based on the modified Dumas Method (FlashSmart, Thermo Fisher Scientific, MA, United States). The elemental analyser consisted of two furnaces, one for C/H/N/S measurements at 950 degrees and the other for oxygen measurement at 1060 degrees. A 140 mL/min carrier gas, a 250 mL/min oxygen gas, and a 100 mL/min reference gas were used.

PHB was extracted from the dried and ground biomass solids in 13 mL dimethyl carbonate (DMC, Sigma-Aldrich ReagentPlus®, 99%) to target a maximum theoretical polymer concentration of nominally 20 mgPHB/mL but lower in some cases due to limitations in the available amount of sample. 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 a 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 RCF over 2 min) with pre-heated (90 °C) tube inserts to remove the 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 (Sartorius MA37). The 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 efficiencies were calculated relative to the amount of biomass in the extraction tube after the initial re-drying before solvent addition. The polymer film thermal properties were characterised by TGA and DSC (as described below).

The dried ground biomass solids and the extracted PHB films were assessed by TGA (TGA 2, Metller Toledo, Switzerland) for biomass PHB content and thermal stability (Chan et al., 2017). Representative subsamples of about 5 and 2 mg of dried biomass or recovered polymer were used, respectively. The method includes the estimation of sample residual moisture/solvent and PHB contents as well as the overall dried sample organic and inorganic fractions. Briefly, pre-weighed samples were inserted into the TGA at 80 °C with nitrogen purge gas at 50 mL/min. The temperature was increased (10 °C/min) to 105 °C and held for 15 min drying, wherein removed moisture (or residual solvent) weight could be estimated. Temperature was increased (10 °C/min) to 550 °C and held for 30 min. PHB 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 from the weight lost after incubation at 550 °C with air atmosphere after 30 min. The biomass PHB content as gPHB / gVSS was derived from the background corrected characteristic polymer decomposition peak detectable from the derivative thermogravimetric trend as described in Chan et al. (2017) using in-house Matlab data processing algorithms (MathWorks, MA, USA). Non-PHB biomass (NPB) was estimated as VSS minus PHB mass. Subsequently, the mass ratio of PHB and non-PHB biomass (f_{PHB}) was calculated. Reference samples included PHA-rich biomass with known polymer content (45.1 ± 0.6 gPHA/gVSS), and pure PHB (>98% purity, Biomer, Germany). Instrument temperature measurement was calibrated based on Curie temperature with a nickel standard following Mettler-Toledo methods.

Differential scanning calorimetry (DSC 3+, Mettler-Toledo, Switzerland) of the polymer in the dried biomass and extracted films was performed based on previously described methods (Chan et al., 2017). About 5 and 2 mg dried biomass and recovered polymer were used from each sample, respectively. Weighed samples in vented crucibles 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 crystallisation enthalpies were estimated with respect to the mass of PHB in the sample estimated from TGA measurements for the same samples. Reference samples included pure PHB (>98% purity, Biomer, Germany), and an in-house poly(3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV standard (34% wt. 3-hydroxyvalerate content). The instrument was calibrated with pure zinc and indium standards according to Mettler-Toledo methods.

Statistical analyses, including multilinear regression and t-tests, were performed with Graphpad Prism (Version 9).

3. Results and discussion

3.1. Incubation and preservation of accumulated PHB quantity

Three batches of pilot scale PHB-rich biomass were produced. The measured maximum biomass polymer contents at the end of 20 h of accumulation were 0.37, 0.53 and 0.50 gPHB/gVSS, respectively. Each batch of the PHB-rich biomass was characterised by elemental analysis to obtain a representative estimate of the NPB COD content for making COD mass balances. Acetic acid results in the accumulation of PHB, and the elemental composition of PHB is ($C_4H_6O_2$). For PHB, 1.67 gCOD/gPHB is applied. Combining information of VS, and PHB contents with elemental analysis, the non-PHB biomass compositions were estimated and found to be consistent between batches as shown in Table 1. The average COD of the NPB was found to be 1.36 gCOD/gNPB and this value was applied in the COD mass balance evaluations. The NPB composition and theoretical COD conversion factor are similar to what was reported in the literature ranging from 1.37 to 1.48 gCOD/gNPB (Chen et al., 2020).

Trends in development for biomass PHB content are typically expressed as PHB concentration over the total VSS concentration during accumulation in the research literature. This ratio is indicative of the performance of the active biomass during an accumulation process towards reaching a steady relative level of the accumulated polymer in the biomass. It is the value measured directly by TGA. Alternatively, the PHB to NPB ratio shows trends of the relative fate of PHB with respect to NPB. Biomass PHB content is converted to a PHB to NPB ratio as follows:

$$f_{PHB} = \frac{\text{PHB mass}}{\text{NPB mass}} = \frac{\text{PHB content}}{\text{1-PHB content}} \quad (1)$$

Table 1
The VSS and PHB contents of the biomass TSS, and the estimated non-PHB biomass (NPB) elemental composition were used towards calculating the corresponding NPB COD conversion coefficient from three distinct batches of PHB-rich biomass.

	VSS/TSS %	PHB content gPHB/gVSS	NPB composition	Conversion coefficient gCOD/gNPB
Batch 1	88.93	0.37	$CH_{1.7}N_{0.17}O_{0.55}$	1.35
Batch 2	90.76	0.53	$CH_{1.7}N_{0.18}O_{0.51}$	1.36
Batch 3	87.47	0.50	$CH_{1.7}N_{0.20}O_{0.48}$	1.37
Average				**1.36**

The PHB to NPB ratio on a COD basis was estimated with the conversion factors for PHB (1.67 gCOD/gPHB) and for NPB (1.36 gCOD/gNPB) from the elemental analyses.

The PHB to non-PHB biomass ratio (f_{PHB}) reported on the relative gain or loss of PHB with respect to non-PHB biomass solids during aerobic or anaerobic incubations. Typical results of trends are shown for the PHB to NPB ratios in the set of incubations for initial pH 7 for aerobic or anaerobic conditions at selected temperatures (Fig. 1). Similar plots of results for the other initial pH sets of incubations are provided as supplementary information (Figure S3 and Figure S4).

Under aerobic conditions without further exogenous substrate supply after PHB accumulation, intracellular PHB is expected to be consumed. The trends of consumption, as shown in Fig. 1 and SI were fitted to the model of a shrinking particle with least squares regression analysis. In this model, PHB removal rate decreases asymptotically following a 2/3 power law as a function of f_{PHB} (Tamis et al., 2014). The integrated rate function solved for f_{PHB} is as follows:

$$f_{PHB} = (\sqrt[3]{f_0} - \frac{1}{3}kt \cdot \frac{C_{x0}}{C_x}^{\frac{1}{3}})^3 \quad (2)$$

where f_{PHB} is PHB to non-PHB biomass ratio (COD basis); f_0 is the initial PHB to non-PHB biomass ratio; $\frac{C_{x0}}{C_x}$ is the ratio between the initial and final non-PHB biomass. For fitting the model, Eq. (2) was linearised and the experimentally determined average f_{PHB} and $\frac{C_{x0}}{C_x}$ values from replicate experiments as a function of time were used to estimate the rate constant k and intercept f_0 by linear regression analysis, Eq. (3):

$$\sqrt[3]{f_{PHB}} = \sqrt[3]{f_0} - k\left(\frac{1}{3}t \cdot \frac{C_{x0}}{C_x}^{\frac{1}{3}}\right) \quad (3)$$

As illustrated in Fig. 1(a) and SI, and similarly for all the initial pH conditions and temperatures with aerobic incubation, the shrinking particle model represented the decay of PHB mass to negligible levels. The aerobic groups served as the benchmark for the polymer fate due to an expected depolymerase activity stimulated by famine conditions and influenced due to different environmental conditions of initial pH and temperature (Tamis et al., 2014).

The estimated rate constant k from the model distinguished between levels of depolymerase activity due to initial pH and temperature (Table 2). Rate constant k ranged from 0.19 to 0.55 d^{-1} depending on the initial pH and temperature. The k value for the initial pH 7 and 25 °C was similar to the k value reported by Koller et al. (2015) in which pure culture PHA-rich biomass was incubated aerobically at pH 7 and room temperature. In the current study, alkaline pH consistently exhibited relatively higher k values for any given incubation temperature. For initial pH 5.5 and 7 groups, the rate constant k increased when the temperature increased from 25 °C to 37 °C and then decreased when the temperature further increased to 55 °C. For the initial pH 10 group, the rate constant k increased from 25 °C to 37 °C then remained at a similar level when the temperature further increased to 55 °C. Within three temperature conditions, the maximum depolymerase activity is the highest between 25 and 55 °C. Mild acidic conditions and elevated temperatures also stimulated higher depolymerase activities relative to neutral initial pH conditions. Therefore for an activated sludge from a municipal wastewater treatment plant with operating pH between 7 and 8, and temperatures between 15 and 30 °C, mild stresses in pH and temperature, in combination with oxygen supply, are detrimental to the objectives of polymer product conservation (Henze, 1997). However, if aeration cannot be avoided, then acidic conditions and lower temperatures are preferred.

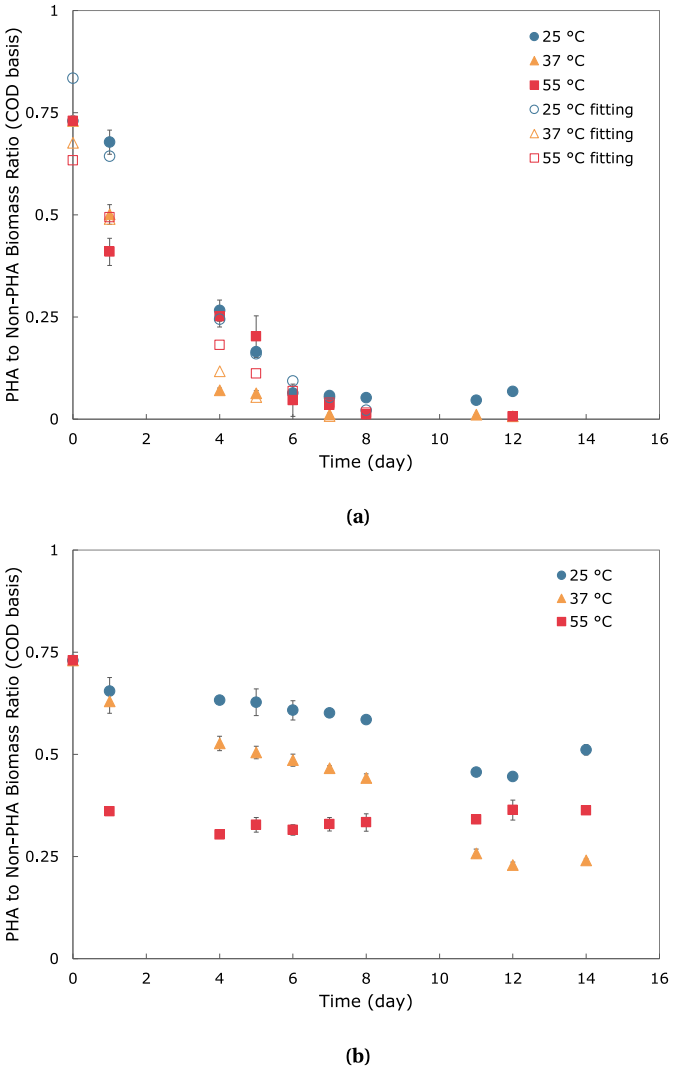

Fig. 1. The trends of PHB to non-PHB biomass ratio (f_{PHB}) over time for an initial pH of 7 with incubations at 25, 37 and 55 °C aerobically (a) and anaerobically (b). Error bars report the standard deviation between 3 replicate samples from respective incubation tubes. Symbols cover error bars in some cases.

Table 2
The rate constant k of aerobic incubation (d^{-1}) for polymer degradation at different initial pH and temperature derived by the shrinking particle model described by Eq. (2) (Tamis et al., 2014) with standard errors of the average rate estimated based on Eq. (3) and from the triplicate experiments.

	Initial pH 5.5	Initial pH 7	Initial pH 10
25 °C	0.19 ± 0.01	0.24 ± 0.03	0.36 ± 0.02
37 °C	0.54 ± 0.07	0.28 ± 0.03	0.54 ± 0.04
55 °C	0.35 ± 0.06	0.19 ± 0.03	0.55 ± 0.08

Table 3
The PHB degradation rate (%/day) over the first day under anaerobic conditions estimated using the average normalised PHB COD from triplicate experiments on day 0 and day 1.

	Initial pH 5.5	Initial pH 7	Initial pH 10
25 °C	0.13	0.17	0.50
37 °C	0.25	0.17	0.70
55 °C	0.32	0.63	0.42

Using the same starting PHB-rich biomass, the incubations under strictly anaerobic conditions showed a different kind of characteristic trend (Fig. 1(b)) compared to the aerobic benchmark cases (Fig. 1(a)). Since the polymer metabolism due to depolymerase activity requires energy, anaerobic conditions under famine were expected to inhibit excessive polymer loss as predicted by the aerobic model described by Eq. (2). However, on the contrary, an initial loss in the relative amount of PHB was observed over the first day before the PHB level with respect to non-PHB biomass became stabilised over at least 8 days of continued incubation.

Even under anaerobic conditions, a limited amount of the accumulated polymer became rapidly metabolised. To estimate for differences in the effect of environmental conditions on the initial relative anaerobic PHB loss, an average degradation rate over the first day was estimated for f_{PHB} and is summarised in Table 3. The activated sludge used in the present study was a nitrification–denitrification biomass. This activated sludge is anticipated to be dominated by obligate or facultative aerobic microorganisms. Therefore, anaerobic conditions would be expected to inhibit depolymerase activity due to a lack of energy flow to kick off the metabolism (Jendrossek, 2009; Oeding and Schlegel, 1973; Ong et al., 2017). Lower temperatures and neutral or mild acidic conditions offered the greatest degree of polymer preservation. One of the possible reasons for higher or lower initial albeit limited degradation rate under anaerobic conditions could be due to an imposed environmental stress on the freshly accumulated PHB-rich biomass. To deal with stress, microorganisms may metabolise PHA (Müller-Santos et al., 2021). This ability and extent of losses seem to be further influenced by initial pH and temperature. Under alkaline conditions, rates of loss are indicated to be highest between 25 and 55 °C. However, under neutral or mildly acidic conditions, the extent of loss is observed to have increased with temperatures up to 55 °C, and decreased with lower pH. These results support that lower pH is preferred to limit the extent of polymer degradation.

The PHB to non-PHB biomass f_{PHB} indicated the depolymerase activity level and the relative change of PHB. If anaerobic incubation were effective in solubilising NPB, then f_{PHB} would be expected to increase in time. However, in the balance after initial PHB losses and up to 8 days of incubation, the amount of PHB relative to NPB remained stable.

A COD mass balance on PHB was applied to determine if this stability corresponded to the absolute or relative preservation of the accumulated polymer in the PHB-rich biomass. The concentration of polymer for each sampling time was calculated from the COD mass balance:

$$tCOD = sCOD + COD_{NPB} + COD_{PHB} \quad (4)$$

where tCOD and sCOD were measured, and the COD ratio of PHB and NPB were determined from TGA measurements coupled to PHB and estimated NPB COD content per unit mass. Since the three different accumulation batches started with different absolute PHB concentrations, relative PHB concentrations with respect to the initial level are reported enabling a direct comparison of trends (Fig. 2, Figure S5 and Figure S6).

For the aerobic group, trends of PHB concentration (Fig. 2(a), Figure S5 and Figure S6) reflect the trends of f_{PHB} (Fig. 1(a)). Over 2 weeks, the amount of the polymer decreased to negligible levels (<4.5% of initial PHB) irrespective of environmental conditions applied. Aerobically, PHB is expected to be hydrolysed into 3-hydroxybutyrate and then converted to CO_2 (Jendrossek and Handrick, 2002).

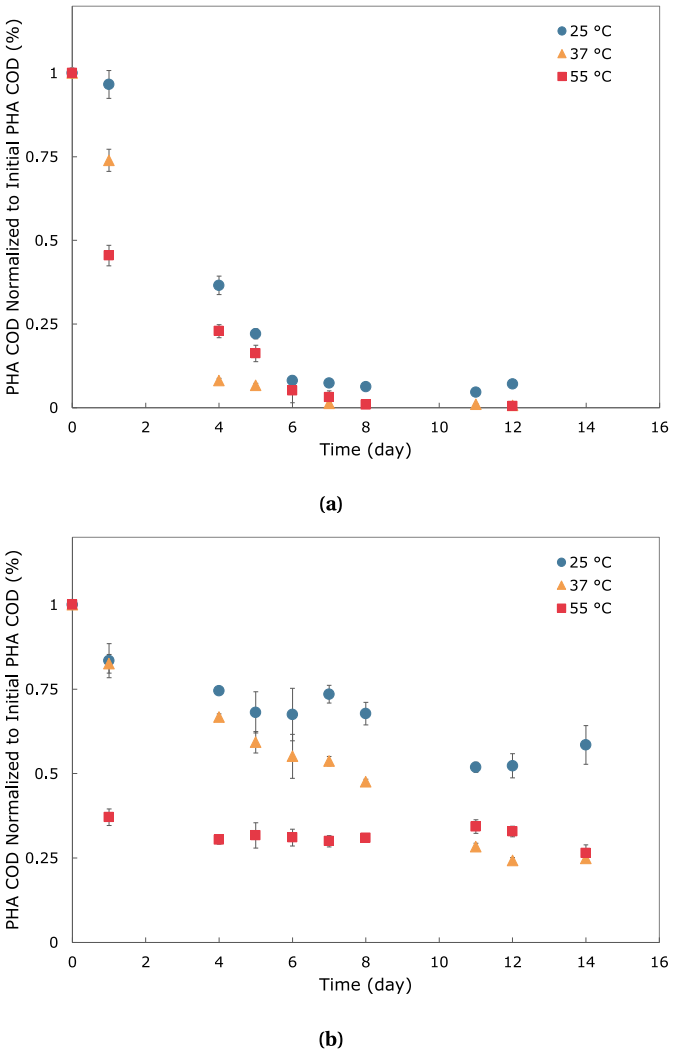

Fig. 2. The trends of PHB concentration (normalised to the initial concentration) for the experimental groups with an initial pH of 7 and incubated at different temperatures (25, 37 and 55 °C) aerobically (a) and anaerobically (b). Error bars report the standard deviation between 3 replicate samples from respective incubation tubes. Symbols cover error bars in some cases.

PHB levels under anaerobic conditions (Fig. 2(b), Figure S5 and Figure S6) exhibited an initial rapid decrease of up to 82% of PHB COD within the first few days. In control experiment 2, PHB powder was incubated with water at acidic, neutral and alkaline pH for more than 30 days, and no 3-hydroxybutyrate or VFAs were measured. This confirmed that the PHB hydrolysis was slow without microbial activity present. It also supports that the initial rapid PHB loss was due to biological activity. After the fast initial degradation, the polymer amount stabilised. Therefore, both PHB concentrations and NPB concentrations remained constant. This observation is similar to what was reported by Shamsul Huda et al. (2013) and Huda et al. (2016). Methanogenesis onset was observed for the cases of initial pH of 10 at 37 and 55 °C after about 8 days. With the onset of methanogenesis, PHB losses resumed.

Losses over time during anaerobic incubation of both PHB and NPB suspended solids may suggest lysis of some PHB-containing biomass, as it was also discussed by Huda et al. (2016). Selective staining and microscopy methods have revealed different morphotypes of PHA storing microorganisms also expressing differences in cell rigidity (Pei et al.,

2022b). Not all cell types will be equally robust to environmental stress, and this is, therefore, a challenge for downstream processing of mixed culture biomass. Optimal conditions for polymer quality preservation and recovery for some morphotypes may result in undue losses or, conversely, reduced yields for recovery from other morphotypes.

Anaerobic conditions with exogenously supplied PHB in control experiment 1 resulted in rapid complete removal of added polymer powder after an initial lag phase with an initial pH of 7 (Figure S7). The lag phase was longer, and the degradation rate was slower, given an initial pH of 10. The lag phase was also prolonged with an initial pH of 5.5. Exogenous PHB was readily degraded as illustrated by control experiment 1 (Figure S7), and endogenous PHB remained stable for several days under anaerobic conditions, notwithstanding conditions that promoted an initial degree of polymer metabolisation. Thus, at least a fraction of the PHB-rich biomass can remain robustly intact, and stored polymer contents can be preserved anaerobically. Exploration for mechanisms and methods for mitigating the observed metabolisation of polymer mass is part of the ongoing investigation.

The main fermentation products during anaerobic incubation were acetate, butyrate and propionate. PHB degradation is understood to follow steps starting with depolymerase activity releasing 3-hydroxybutyrate. However, no build-up of 3-hydroxybutyrate was measured, only volatile fatty acid formation similar to the results reported by Huda et al. (2016). Soluble hydrolysis products may be released if host cells hydrolyse an excess of PHB and cannot metabolise it further under anaerobic conditions. These soluble products are readily fermented into acetate and butyrate. The theoretical levels of acetate and butyrate formation were estimated based on loss of PHB and the expected stoichiometry $2CH_3CHOHCH_2COO^- \rightarrow 2CH_3COO^- + CH_3(CH_2)2COO^- + H^+$ for the extracellular depolymerase activity as reported by Stieb and Schink (1984). In the control incubation experiment 1, the exogenous supplied PHB formed acetate and butyrate following the stoichiometry reported by Stieb and Schink (1984). For the PHB-rich biomass incubations, as shown in Table S1, the predicted levels of butyrate were similar to the measured butyrate concentration. For acetate, the measured concentration was higher compared to the predicted levels. These results suggested that butyrate formation was due to PHB fermentation. The acetate levels were due to both PHB and NPB fermentation during anaerobic incubations (Shamsul Huda et al., 2013; Huda et al., 2016). The propionate only came from NPB fermentation. Confirmation of the interpreted PHB transformation would require further experiments. One approach could be using stable isotopes as tracers. The fermentation products during PHB-rich biomass anaerobic incubation experiments suggested that intracellular PHB or the metabolites of PHB may become released to the matrix and are rapidly fermented. In control experiment 3 where PHB powder was incubated with commercially available lipase enzyme, an increasing level of 3-hydroxybutyrate was observed (Figure S8). This suggests that extracellular PHB hydrolysis can be carried out by generic enzymes that react to ester bonds.

Anaerobic conditions naturally preserved the accumulated PHB for a period of time better than aerobic conditions. However, even in the best case scenario (initial pH 7 and 25 °C), around 42% of the polymer was lost within the experimental period. Therefore, for temporary staging with storage or transportation of the PHB-rich biomass, anaerobic conditions alone are not sufficient, and results support that lower temperatures or pH levels need to be applied shortly after the accumulation process is terminated. In previous work up to pilot scale, PHB-rich biomass acidification down to pH 2 has been applied to induce higher polymer thermal stability before final dewatering and drying (Werker et al., 2020). However, even for this lower pH acidification treatment and wet biomass storage at 4 °C, molecular weight loss still occurred at a quite slow but measurable rate. Selective staining with confocal microscopy evaluation was applied to PHB-rich biomass incubated anaerobically with initial pH of 5.5 at 55 °C over 14 days. As shown in Fig. 3, the selective staining revealed a dominance of intact

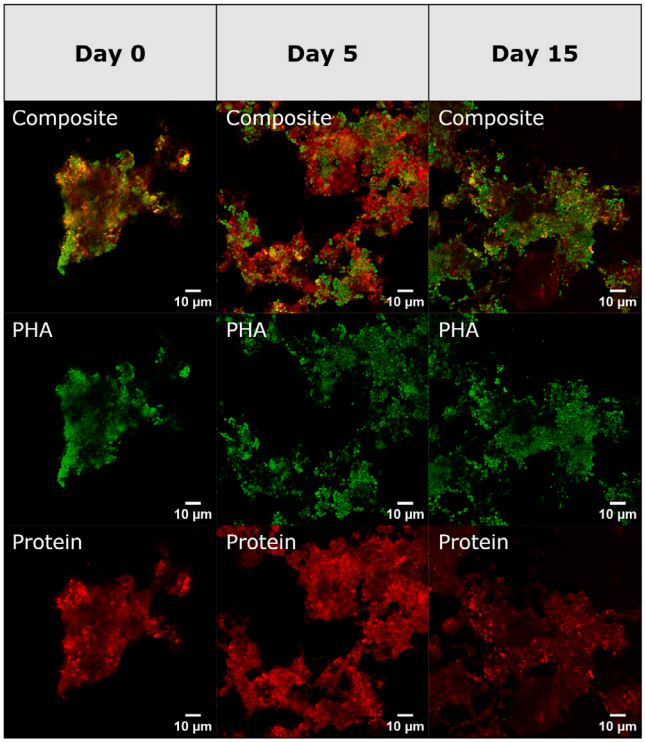

Fig. 3. The selective staining of PHB (green) and non-PHB biomass (green) for one of the experimental groups under the anaerobic condition with initial pH of 5.5 at 55 °C.

cell structures containing PHA granules (Pei et al., 2022b). Therefore, preserved PHB presented as still being intracellular after the anaerobic incubation (Pei et al., 2022b).

Neglecting the initial anaerobic PHB degradation, intracellular PHB preservation was found to be possible due to a lack of oxygen supply. Intracellular depolymerase activity levels were influenced by pH and temperature but were not prevented when oxygen was supplied. It has also been reported that environmental conditions can cause PHA granules to coalesce and become disrupted as functioning carbonosomes (Sedlacek et al., 2019b). The crystallisation of the polymer is reported to ensue upon dehydration, but before that, the polymer in a hydrated amorphous structure may form crystalline associations like a gel which could impede the intracellular depolymerase activity (Porter and Yu, 2011b; Obruca et al., 2017). Meanwhile, the granules within intact cells may remain, kept away from extracellular depolymerases, which were anyway found to be inhibited by acidic conditions.

3.2. Incubation and changes to accumulated PHB extractability

The observed trends of PHB concentration illustrated in Fig. 2(b) could be a mixture of effects, including discussed changes in granule structure or function, *in-situ* polymer crystallisation, depolymerase activity attenuation with time, and cell function losses due to prolonged anaerobic incubation. Increased temperatures to the thermophilic range are reported to induce changes in polymer morphology but will also increase depolymerase activity levels. Effects of pH on inferred *in-situ* PHA crystallisation have not been conclusive (Porter and Yu, 2011b,a). A higher crystallisation rate with alkaline conditions than neutral and acidic pH has been reported, but results of opposite trends with pH are also published (Sedlacek et al., 2019a; Porter and Yu, 2011a). It is not a trivial matter to measure for changes in the polymer nature *in-situ* without altering the polymer as a consequence of the measurement approach. Exploration of approaches is part of the ongoing investigation. The present study processed biomass samples using a

standardised acidic wash and thermal drying protocol. Even if sample processing influences the polymer nature, it was interesting to observe if the incubation with different applied environmental conditions would result in systematic trends of polymer quality in the biomass.

Differences in polymer quality due to the incubation were assessed based on thermal decomposition temperature (T_d), polymer melt enthalpy (ΔH_m), and extractability. Thermal properties were assessed for the polymer in the dried biomass and for the extracted polymer. An advantage of PHB for the present investigation is that the homopolymer normally exhibits a relatively high crystallinity and crystallisation rate (Laycock et al., 2014). This behaviour enables the evaluation of the melt and crystallisation behaviour of the polymer even in dried biomass samples. Results are summarised in Table 4.

Acidic washing of the PHA-rich biomass is a method to enhance thermal stability (Chan et al., 2017). The T_d for the PHB in the biomass was consistent at 286±2 °C. Incubation did not influence the ability to harmonise for similarly enhanced polymer thermal stability before solvent extraction. Neglecting a measurement outlier (pH 7 and 55 °C), extraction resulted in recovered PHB with higher average thermal stability 297±1 °C as is expected. NPB has been shown to influence polymer thermal stability (Kopinke et al., 1996).

PHB melt peaks and enthalpies were quantified for the second heating ramp after a first melt and quench at −10 °C/min cycle to standardise the sample thermal history. Typical examples of the melting curve measured by DSC for the PHB standard, PHB in biomass and PHB films are shown in Fig. 4. The melt peak temperature was estimated (T_m), and the peak melting enthalpy was referenced to the amount of PHB in the weighed sample. The melting temperature for the polymer in the biomass at the beginning and the end of the incubation did not show a significant difference based on independent samples *t*-test (p = .37). The average melting temperature for the polymer in the biomass values (T_m = 175±3 °C, N = 12) were higher compared to the reference PHB (T_m = 170 °C). The extracted film melting temperatures before incubation (T_m = 176±2 °C, N = 3) were significantly higher compared to the film after incubation (T_m = 169±1 °C, N = 9) based on independent samples *t*-test (p < .05). The average extracted film melting temperatures before incubation (T_m = 176±2 °C) was also higher compared to the reference PHB (T_m = 170 °C). In contrast, the extracted film after incubation (T_m = 169±1 °C, N = 9) showed similar melting temperatures compared to the reference PHB (T_m = 170 °C). Before incubation, the melting temperature showed no significant differences between the polymer in biomass and the extracted films (p = .50). However, after incubation, the melting temperatures for the polymer in the biomass (T_m = 172±3 °C, N = 9) were on average 3 °C significantly higher compared to the extracted PHB film (T_m = 169±1 °C, N = 9) based on independent samples *t*-test (p < .05). The systematic average difference of melt temperatures for the polymer in the biomass versus in the extracted films can be an artefact of differences in heat transfer properties (film versus powdered PHB-rich biomass), resulting in a lag in heat flux to and from the polymer in the biomass (Menczel and Prime, 2008). Different incubation conditions were not found to significantly influence (95%CI) the average distributed polymer inclusion melting temperatures as expressed in the biomass in any systematic way and with respect to the standardised sample treatment protocol. An average decrease in melt temperature for the extracted polymer after the incubation does indicate that the extracted polymer thermal properties, now as a blended mixture, were changed by the incubation.

The polymer melt enthalpy in the biomass was on average lower and more variable than for the corresponding extracted polymer (ΔH_m = 49±22 J/gPHB for PHB in biomass versus ΔH_m = 90±8 J/gPHB for extracted PHB). Variability of the extracted PHB melt enthalpy was not correlated to the recovered PHB purity, which ranged from 42 to 94 wt percent PHB. The reference PHB expressed a melting enthalpy of 98 J/gPHB. Melting temperature and melting enthalpy depend on the crystalline lamellae thickness. This thickness varies with crystallisation conditions (Laycock et al., 2013). Crystallisation conditions for the polymer in all samples compared were the same based on the second DSC melt and quench cycle. Therefore, the expressed changes for polymer in-biomass average melt enthalpy, and extracted polymer melting temperature were interpreted to reflect significant effects on polymer properties due to the incubation. The significance and fundamental interpretation of these effects require further investigation.

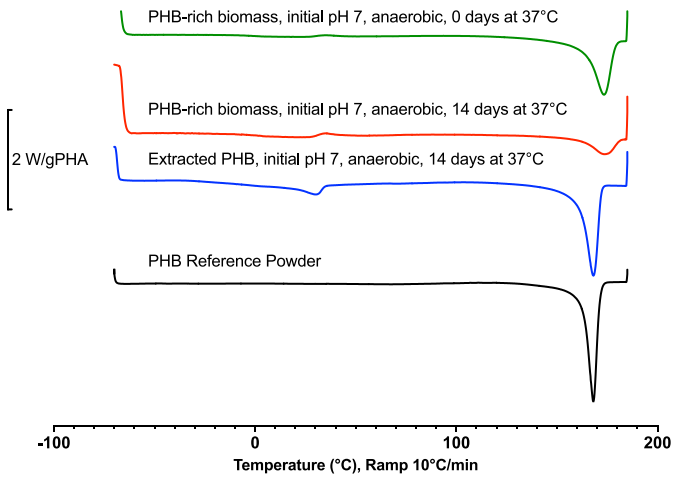

Fig. 4. Melting of the standard PHB, PHB-rich biomass, and extracted PHB film measured by DSC.

The variability of the expressed PHB melt enthalpy for the polymer in the biomass could be coupled and correlated to the incubation conditions. In general, higher PHB-in-biomass enthalpies were found given before incubation, lower incubation temperatures, or lower incubation pH conditions (Table 4). The melt enthalpy of semi-crystalline polymers relates to the degree of crystallinity with respect to the thermal history of the sample. PHB is expected to be able to reach a crystallinity of 60 percent (Laycock et al., 2013). While the direct estimation of polymer crystallinity from melt enthalpy measurements cannot be made with certainty, relative differences are indicative.

A high relative crystallinity for PHB compared to the random copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), makes it a more challenging polymer to extract, especially when using so-called PHA-poor solvents (Werker et al., 2020). Longer extraction times or higher extraction temperatures are necessary to reach high extraction efficiencies for PHB, with all other things being equal. The PHB extraction efficiencies are shown in Table 4. The uncertainty of extraction efficiencies is considered to have come from the uncertainty of polymer content measurement in biomass which has been estimated to be around 2% (Chan et al., 2017). The lower the PHA content of the biomass, the more sensitive the mass balance was for measurement uncertainty of the biomass PHA content. The one main outlier is the final sample incubated at 25 °C and pH 10. This sample has a PHA content estimated at 17.5% (Table S2). If this value were to be increased to 19%, the outcome would give a 100 percent extraction efficiency. Thus this outlier is understood to be effectively 100 percent polymer recovery. From the extraction mass balance evaluations, an average 77 ± 19 percent of PHB was extractable from the PHB-rich biomass samples. Of notable interest from multilinear regression analysis of the extraction data (Table 4) was the finding that variability in extractability was positively correlated to the measured PHB-in-biomass melt enthalpy (p = .0023). Therefore, the greater the expressed potential for the PHB in the biomass to crystallise, the better was the PHB extractability. The sampling protocol, involving an acid wash, thermal drying, and a melt and quench cycle before the melt enthalpy evaluation cannot represent the property of the PHB *in-situ*. However, the standardised sample protocol still permitted observation and measurement for a change

Table 4
The properties of PHB standard, PHB in the biomass, and extracted PHB from single measurements of the representative samples for each experimental group.

pH	T (°C)	Time	Extraction efficiency (%)	gPHB/gTS		T_d[a] (°C)		T_m[b] (°C)		ΔH_m[c] (J/gPHB)	
				Biomass	Film	Biomass	Film	Biomass	Film	Biomass	Film
PHB[d]	–	–	–	>0.98		298		170		98	
5.5	–	0	96	0.48	0.94	287	295	177	176	82	96
5.5	25	End	85	0.25	0.79	288	298	169	171	56	80
5.5	37	End	69	0.32	0.77	287	298	172	169	50	94
5.5	55	End	80	0.44	0.86	287	298	172	168	62	96
7	–	0	100	0.33	0.90	290	297	174	173	68	83
7	25	End	81	0.28	0.85	287	298	173	169	48	72
7	37	End	43	0.15	0.54	285	296	169	168	21	97
7	55	End	69	0.20	0.68	285	285	174	169	32	94
10	–	0	71	0.48	0.92	287	298	171	178	59	92
10	25	End	108	0.18	0.75	288	299	173	169	66	92
10	37	End	65	0.08	0.46	284	296	178	166	27	101
10	55	End	52	0.08	0.42	283	295	170	168	11	89

[a] T_d is the decomposition temperature for PHB at a heating rate of 10 °C/min.
[b] T_m is the melting temperature for PHB.
[c] ΔH_m is the melting enthalpy of the PHB.
[d] PHB standard acquired from Biomer with a purity >98%.

in polymer quality that had an interpreted bearing on the polymer extractability. These results suggest that recovery yields in the DSP are influenced by the condition of the polymer in the biomass, which can be affected by the applied methods of recovery. pH condition influenced the polymer condition from the start. An acidic pretreatment was found to be preferred for improved extractability.

The recovered polymer was not pure, and because the solvent was evaporated from the extracted solids, the solubilised NPB was also evaluated. On average, 8 ± 2 percent of the NPB was co-extracted with the recovered polymer. Therefore, biomass with lower PHB content yielded an extracted polymer of lower purity for the total extracted solids. Multilinear regression analysis suggested that less NPB was extracted given lower temperature incubations (p = .0367). Higher incubation temperatures solubilise more NPB (Table S1) and may result in solvent soluble NPB. The solvent soluble NPB will be extracted and will follow in the solvent as potential contaminants in the final extracted polymer. Post extraction washing steps to remove extracted contaminants add further cost to the recovery process. Therefore, PHB-rich biomass pretreatment and its impact on the total extract polymer purity require further systematic fundamental and techno-economic evaluations towards optimised DSP economy.

4. Conclusions

- Intracellular depolymerase activity for a mixed culture PHB-rich biomass can be constrained by anaerobic conditions but changes in environmental conditions may nevertheless cause significant polymer losses even for short term storage (up to 48 h).
- Extracellular depolymerase activity for a mixed culture PHB-rich biomass is limited for an extended period of time so long as the PHB remains intracellular.
- Acid environmental conditions are preferred to preserve quantity and quality for recovery of PHB from PHB-rich biomass following a mixed culture accumulation process.
- The melting enthalpy of PHB in the biomass following a standardised sample workup can be indicative of the PHB extractability from the biomass.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.watres.2023.119653.

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