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Demonstrating performance in scaled-up production and quality control of polyhydroxyalkanoates using municipal waste activated sludge

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ABSTRACT

Significant progress has been made over the past decade with pilot scale polyhydroxyalkanoate (PHA) production by *direct accumulation* using municipal waste activated sludge (WAS). However, industrial upscaling experiences are still lacking in the research literature. In this study, a demonstration scale (4 m^3) PHA production process was operated using industrially relevant equipment and compared favourably to those from parallel pilot scale (200 L) production runs. WAS grab samples from a Dutch full scale municipal wastewater treatment plant (WWTP) was used as the biomass source. Final biomass PHA contents and production yields, that are critical for technology viability, were statistically the same between the experiments conducted at pilot scale (0.41 ± 0.02 gPHA/gVSS and 0.42 ± 0.02 gCOD/gCOD) and demonstration scale (0.45 ± 0.05 gPHA/gVSS and 0.39 ± 0.07 gCOD/gCOD). The results furthermore aligned with previous 1 m^3 piloting experiences and five year old historical data that similarly used WAS sourced from the same WWTP. Scalability for the technology and a robustness of the applied PHA production methods using WAS were demonstrated. Temperature and foaming control were identified to be critical to upscaled process engineering and design towards successful industrial implementations. The results of the present study, combined with previously produced PHAs and those historical data, support that feedstock quality predictably determines both the average PHA co-monomer content, as well as the blend distribution. PHA solvent extraction from WAS is inherently a blending process. Extraction homogeneously mixes polymer contributions from collectively stored granules from all species of microorganisms in the biomass. Dried PHA-rich biomass batches can be stockpiled and batches can be blended in extraction processes for both recovery and formulation to reach consistent polymer qualities across production batches. More centralized extraction facilities are therefore anticipated to offer economic benefits due to scale and greater opportunities for product quality specification and control. Research findings are presented herein of the production scale comparative study along with practical perspectives of technological readiness for realizing WAS based industrial scale PHA production, quality control, and the supply chains that will be necessary for successful commercial implementation.

1. Introduction

Polyhydroxyalkanoates (PHA) are a class of biodegradable polymers that are produced by a wide range of microorganisms as intracellular granules (Dawes and Senior, 1973). Granules are temporarily stored carbon for microorganism survival in dynamically changing environments. Such changing environments can favour the selection of PHA-accumulating microorganisms in a mixed microbial culture (MMC) biomass (Van Loosdrecht et al., 1997; Reis et al., 2003). Selective dynamic environments can occur in nature but they are also inherently present in bioprocesses used for municipal biological wastewater treatment. Municipal waste activated sludge (WAS) is therefore commonly found to be enriched with PHA accumulating microorganisms (Van Loosdrecht et al., 1997; Pei et al., 2022a). An activated sludge that is enriched with PHA accumulating microorganisms can be an exploitable renewable resource. It is the input functional

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biomass for *direct accumulation* bioprocesses used for PHA production as recently reviewed in the literature (Estevez-Alonso et al., 2021a).

Commercial quality PHAs can be produced with municipal WAS given availability of volatile fatty acid (VFA) rich waste streams as substrates. The produced PHAs are typically co-polymer blends of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or PHBV (Bengsston et al., 2017; Werker et al., 2020; Estevez-Alonso et al., 2021a; Pei et al., 2023a). PHBVs are semi-crystalline polyesters and can offer several advantages over conventional fossil based polymers, including biodegradability, bio-compatibility, alongside chemical–physical properties that are suitable for many kinds of applications requiring thermoplastic-like materials (Raza et al., 2018; Estevez-Alonso et al., 2021a). Despite a well-recognized potential of PHBV, steps to scale up production from laboratory to commercial scale are still limited. One major uncertainty posing investment risks for upscaled PHBV production is the limited and unpredictable availability of commercial PHBV supplies today. Significant quantities (e.g., > 5000 tons of PHA per year) of specific PHA grades are essential for advancing plastic formulations in applications where biodegradability is beneficial (Bauchmüller et al., 2021). However, even during prototype development, investment risks persist due to uncertainties around the future availability and qualities of specific commercial-grades, given currently limited supply of pre-industrial scale feedstocks and PHA grades produced therefrom. This is a dilemma that hampers progress towards implementing viable biobased value chains (Estevez-Alonso et al., 2021a). Commercialization risks can be lowered with evidence from well-documented knowledge developments in technical feasibility, as well as disclosed positive experiences in scaled up activities that include results and insights for the up-scaled production polymer quality control.

Several examples of technological feasibility for MMC PHA production at pilot scale have been reported (Estevez-Alonso et al., 2021a). PHBV production using WAS with *direct accumulation* was found to meet criteria for commercial quality at pilot scale with bioreactor volumes of 1 m^3 (Werker et al., 2020). However, piloting production with nominally 1 m^3 production volumes may typically only result in about 1000 g polymer contained in a dried PHA-rich biomass per cycle (around 24 h). Case studies that relate experience in the next step of demonstration scale activities with production volumes that can furnish enough pre-industrial PHBV for prototype product developments are lacking in the research literature. There are always challenges to industrialize methods and processes of technologies towards supplying relevant quantities of pre-commercial products with maintained quality control. Performance limitations due to the inherent process time scales for reaction kinetics and mass transfer rates may be faced alongside sometimes unexpected practical issues, including challenges of material conveyance and management, at every step of scaling up technologies. Therefore, documented systematic demonstration scale studies are necessary to share in supporting progress from proven pilot scale technology feasibility to a security with expectations for practical suitability and likelihood for success in next steps towards rolling out industrial scale operations.

As part of the EU project SCALIBUR (www.scalibur.eu), a bi-weekly routine of *direct accumulation* using municipal WAS was undertaken over 2 years (2019–2020) at pilot 200 L scale and established feasibility to make and recover consistent quality PHBVs of high purity (> 98%). In parallel, industrial scale production performance for *direct accumulation* was benchmarked in a campaign of scaled up batch production using a demonstration (demo) scale bioprocess with volume of 4 m^3. The demo scale process was built to evaluate key industrial scale equipment and to resolve engineering challenges that emerged. Bioprocess methods have been shown to influence outcomes in performance and polymer quality at laboratory scale (Estevez-Alonso et al., 2022a). However, both the pilot and demo scale bioreactors in these projects similarly applied the same respiration control of small input substrate pulse volumes in a fed-batch feed-on-demand bioprocess (Werker et al., 2014, 2022). Therefore, additional experiments were undertaken to make controlled direct comparison between the two bioprocesses with only difference in scale from 200 L to 4 m^3 operations. The comparison objective was to evaluate for any significant influence of scale on PHA production *direct accumulation* performance and the resulting polymer qualities. Specifically, the context of *direct accumulation* bioprocess methods including downstream steps of biomass dewatering and drying were considered.

Replicate PHA production runs were performed in parallel at both pilot and demo scales (November–December 2020). These replicate experiments were made using distinct supplied batches of thickened municipal WAS from Bath WWTP in the Netherlands. This effort to compare for an influence of scale aimed to address whether a supply chain of municipal activated sludge for *direct accumulation* could be expected to yield consistent robust outcomes at commercial scale. The activated sludge biomass performance was considered in isolation of other possible contributing factors that can be due to possible variability from waste feedstock supplies. For the comparative evaluations, PHA was produced using a well-defined substrate comprising acetic and propionic acids as feedstock. Overall outcomes of process performance and polymer properties were monitored and compared between the pilot and demonstration production activities. Outcomes were compared to results from the routine SCALIBUR project production batches and furthermore compared to the experience 5 years earlier of PHARIO where the same source of WAS from Bath WWTP was used in PHBV production campaigns at pilot scale (Werker et al., 2020). To our knowledge, this investigation represents the first critical comparison of scaling up performance for municipal waste activated sludge *direct accumulation* from pilot to demonstration production volumes. Assessments of bioprocess performance including rates and yields as well as recovered PHBV quality from the accumulation campaigns at pilot (200 L) and demonstration (4 m^3) scales were undertaken, and these are reported herein.

2. Materials and methods

2.1. Experimental set-up

Pilot scale production methods were as described previously (Pei et al., 2022b) in a jacketed stainless steel 200 L fed-batch bioreactor. Mixed liquor was continuously recirculated through a 16 L gravity settler connected to the main volume. The process temperature was thermostated at 20 °C by a water bath with recirculation to the reactor jacket. Mechanical mixing was constant at 230 rpm with a standard impeller. Coarse bubble aeration was provided through a membrane disc at a constant flow rate of 50 L/min. Dissolved oxygen (DO), pH and temperature levels were monitored and logged.

Up-scaled demo 4 m^3 PHA production campaigns were similarly performed in a fed-batch bioreactor that was installed in Leeuwarden at the Wetterskip Fryslân WWTP demo site. The bioreactor setup comprised a high-density polyethylene (H/Diameter = 3 m/1.5 m) vessel as depicted in Fig. 1. Outdoor operations with no reactor insulation necessitated some initial process heating during autumn and winter operations. An immersion heat exchanger supplied with boiler water was used. A recirculation loop with spray nozzles at the top of the reactor enabled foam control without necessarily needing to add any anti-foam chemicals. Aeration and mixing were by means of a grid of coarse bubble diffusers at the bottom of the reactor. DO, pH and temperature levels were monitored and logged.

2.2. Biomass source and feedstock

Municipal WAS from Bath WWTP (Rilland-Bath, the Netherlands, 470,000 person equivalents) was used as input PHA accumulating biomass in the pilot 200 L and demo 4 m^3 bioreactors. Bath WWTP treats a municipal and industrial wastewater mix with screening and primary treatment. Secondary treatment is by tanks in series creating

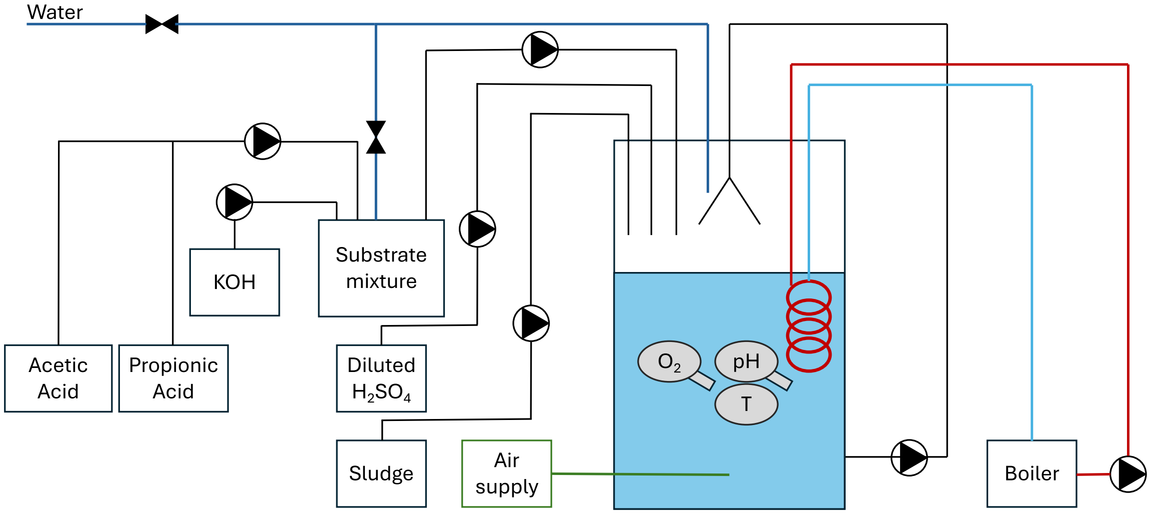

Fig. 1. Schematic drawing of the upscaled demonstration 4 m^3 PHA accumulation bioprocess.

hydraulics towards plug flow in a modified Ludzack–Ettinger activated sludge biological process (10 independent parallel treatment lanes with 20 days solids retention time). Chemical phosphorus removal was performed with $FeCl_3$. Previous work in the project PHARIO demonstrated consistent performance over one year in a routine of campaigns of PHA production by *direct accumulation* with activated sludge from this WWTP (Werker et al., 2020; Pei et al., 2022b).

The WAS supply included all aspects of anticipated industrial scale sludge handling and logistics, including dewatering chemicals for thickening and transport to a remote PHA production facility. Specifically, the secondary WAS grab samples were collected after gravity belt thickening (57.3 gTS/L) and transported from Bath WWTP to Leeuwarden, the Netherlands for *direct accumulation* without any enrichment in advance of the PHA production (Estevez-Alonso et al., 2021a). For routine SCALIBUR project work, thickened WAS was transported in 5 L carboys and stored refrigerated at 4 °C pending its use for pilot accumulations. For demo scale versus SCALIBUR pilot production campaigns, thickened WAS grab samples were transported in IBC containers and used directly without storage for accumulations in parallel at pilot and demo scales.

The PHA production feedstock was a 76:24 mixture (COD basis) of acetic and propionic acids, respectively. Nitrogen (N) and phosphorus (P) were added to achieve a COD:N:P (by weight) of 100:1:0.05 with NH_4Cl and KH_2PO_4. The feedstock was prepared with tap water and the pH was trimmed to 4.5 by stepwise addition of KOH.

2.3. PHA production methods

For each fed-batch PHA production cycle, the pilot or demo reactors were loaded with a pre-determined weight of the gravity thickened WAS and brought to a targeted volume for a starting mixed liquor volatile suspended solids (MLVSS) concentration of about 3 gVSS/L by dilution with tap water. Trends in MLVSS concentration and quality were monitored from grab samples at selected times during the production cycle. Accumulation operation process control was automated in both pilot and demo cases but preliminary and post accumulation steps were undertaken manually and so timed to be performed during normal working hours.

Some initial aeration time was necessary to remove any residual readily biodegradable soluble COD present in the thickened WAS. The reactor, with starting MLVSS, was first aerated for about 12 h. This aeration time was in excess but it was performed overnight due to site access restrictions and logistics. Dissolved oxygen levels trended up to a steady state level indicating steady endogenous respiration for the mixed liquor before the start of an accumulation. The PHA production cycle was manually started in the morning. A production cycle started with a standardized conditioning step referred to as an acclimation (Morgan-Sagastume et al., 2017; Estevez-Alonso et al., 2022a). Conditioning comprised three pulse inputs of substrate and this results in a well-documented consistent positive effect on accumulation response and the final biomass PHA content. Notwithstanding, conditioning did not incur any significant amount of active growth (no enrichment) nor any significant net PHA accumulation (Pei et al., 2023b). The three pulse inputs of substrate during conditioning targeted a maximum of 100 mgCOD/L per pulse. Changes in respiration rate were monitored based on DO. The time for substrate consumption with every pulse input was estimated based on well-defined shift up and down trends in respiration (Werker et al., 2022). Aeration was continued for 3 times the substrate uptake time before giving a next conditioning pulse. The PHA production phase of accumulation was started directly after conditioning.

PHA accumulation was carried out over 20–22 h by fed-batch accumulation with a feed-on-demand strategy wherein substrate was dosed in discrete fixed volume pulses (Werker et al., 2014). Each dosed volume targeted a theoretical peak substrate concentration of 200 mgCOD/L. Feedback control used the respiration response based on monitoring DO as previously described by Werker et al. (2020). For each input of substrate, a downswing and upswing of DO indicated for the response to and exhaustion of added exogenous substrate. The DO upswing trend was used to trigger each subsequent pulse of substrate without undue delay. In this way, a continuous pulse-wise supply of substrate was provided in tact with its consumption. A high respiration rate was consistently maintained while keeping a low average background level of soluble COD in the mixed liquor throughout the production cycle (Werker et al., 2022).

Aeration was stopped at the end of 20–22 h and PHA-rich biomass suspended solids were settled by gravity. Supernatant was decanted and the mixed liquor was acidified to between pH 2 and 2.5 with the addition of H_2SO_4 as previously disclosed (Werker et al., 2013; Pei et al., 2023a). The acidified solids were dewatered by either a filter press (pilot scale operations) or by a decanting centrifuge (demo scale operations). The filter pressing was performed after addition of dewatering chemicals ($FeCl_3$ 77 mg/gTSS and polyelectrolyte (PE, Zetag(R) 9148FS at 1 mg/gTSS)). No dewatering chemicals were used for the demo scale dewatering by centrifugation.

Thickened acidified solids were dried either by a laboratory convection oven (pilot scale operations) or by a commercial drum drier (demo scale operations). Pilot scale oven drying mimicked industrial belt drier operations with manually pressing out the wet cake onto

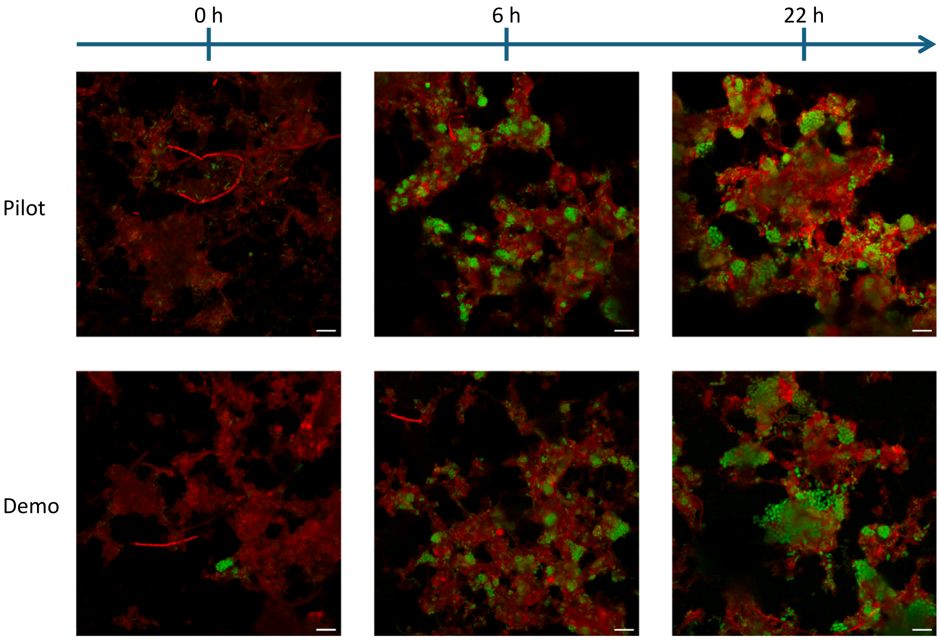

Fig. 2. Visualization of PHA distributions in activated sludge at pilot and demo scales for 0, 6, and 22 h accumulation time by staining PHA with BODIPY (green) and protein SYPRO Red (red). Scale bars represent 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

drying trays to nominally 2 cm slabs and then drying with initially 30 min at 120 °C followed by 100 °C until dry. Demo scale drying was by an industrial steam heated rotating drum drier. Thickened biomass solids were fed onto the heated drum as a thin sheet and flash dried at between 100 and 120 °C with a contact time of less than one minute. In both cases, the harvested dried PHA-rich biomass was further mechanically processed into a granulate suitable for PHA recovery by solvent extraction at laboratory and pilot scales using methods and solvents (dimethyl carbonate and 2-butanol) as previously described (Werker et al., 2020, 2023).

2.4. Analytical methods

PHA production cycle performance was assessed with online measurements (DO, pH and temperature) and mixed liquor grab samples were taken at selected times during each production run. Grab samples were used to assess trends in total suspended solids (TSS) and volatile suspended solids (VSS) based on Standard Methods for solids analyses (Clesceri et al., 1999). A fraction of these grab samples was acidified to pH 2 with addition of 98% H_2SO_4 and then centrifuged at 3248 RCF for 5 min at 4 °C (Beckman Coulter, CA, USA). The pellet was separated from the supernatant and dried overnight at 105 °C. Dried pellets were ground and assessed by Thermal Gravimetric Analysis (TGA 2, Mettler Toledo, Switzerland) for biomass PHA content according to Chan et al. (2017). Polymer quality (thermal properties, monomer composition, intrinsic viscosity) from dried PHA-rich biomass samples was assessed by methods of test tube extraction and analyses (differential scanning calorimetry, pyrolysis gas chromatography with mass spectrometry, solution rheology) as previously described (Pei et al., 2023a; Werker et al., 2023). Commercial pure (> 98%) polyhydroxybutyrate (PHB) powder supplied by BIOMER (Germany) was used as a reference material for DSC and solution rheology measurements.

Development and distribution of accumulated PHBV in the activated sludge flocs was assessed and compared for pilot and demo production runs by methods of fixation, selective staining and Confocal Laser Scanning Microscopy (CLSM). Duplicate grab samples were fixed directly with formaldehyde to a final concentration of 3.7% and preserved in a 1X phosphate-buffered saline and pure ethanol solution at a 1:1 ratio (Llobet-Brossa et al., 1998; Hugenholtz et al., 2002; Pei et al., 2022b). Fixed samples were stored at -20 °C pending staining and imaging. Aliquots of 5 μL fixed sample were loaded in reaction wells (10 per glass slide). Well contents were stained with 0.5 μL of BODIPY 493/503® (Thermo Fisher Scientific, MA, USA) at 2 ng/μL for PHA. In combination 0.5 μL of 100 times diluted Sypro™ Red (Thermo Fisher Scientific, MA, USA) was also applied for staining the non-PHA biomass (Pei et al., 2023b). The glass slides were dried at 46 °C. Residual dye was rinsed from the dried slide with Milli-Q water. Slides were then dried again with compressed air, mounted with VECTASHIELD® HardSet™ Antifade Mounting Medium H-1400-10 and sealed.

The stained samples were evaluated by a Confocal Laser Scanning Microscope LSM 880 (Carl Zeiss, Germany) with Plan-Apochromat 63x/1.4 Oil DIC objectives (Carl Zeiss, Germany). Methods of image capture were as described in Pei et al. (2023b). For each field of view, BODIPY and Sypro Red were excited with Argon laser (488 nm) and a DPSS 561-10 laser (561 nm), respectively. Overlay images were captured into separate image channels. Images were evaluated in Fiji Image J (ImageJ2, Ver 1.52P).

2.5. Data analysis

Biomass PHA content was measured as mass fraction of the volatile suspended solids (gPHA/gVSS). Active biomass (X_a) was estimated as VSS (X_t) minus PHA (X_p) mass. Active biomass was assumed to be represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980). The observed trend for biomass PHA content during PHA production was represented by least squares regression to first order kinetics as in Bengsston et al. (2017):

$$X_p/X_t = A_0 + A_1\left(1 - e^{-k_a t}\right) \tag{1}$$

where A_0 is the initial biomass PHA content, A_1 is the maximum accumulated biomass PHA content, and k_a is the process first order accumulation kinetic constant. The biomass PHA accumulation potential was estimated as the sum of A_0 and A_1. The time $3 \times \tau_a$ ($\tau_a = 1/k_a$ (h)) represented an estimated process time for the biomass to reach 95 percent of the biomass extant maximum PHA accumulation potential.

Specific production/consumption rates and PHA yields on substrate were estimated for the whole accumulation process. The average PHA yields on substrate were calculated on a COD-basis. Polymer COD content was estimated from the measured weight fraction

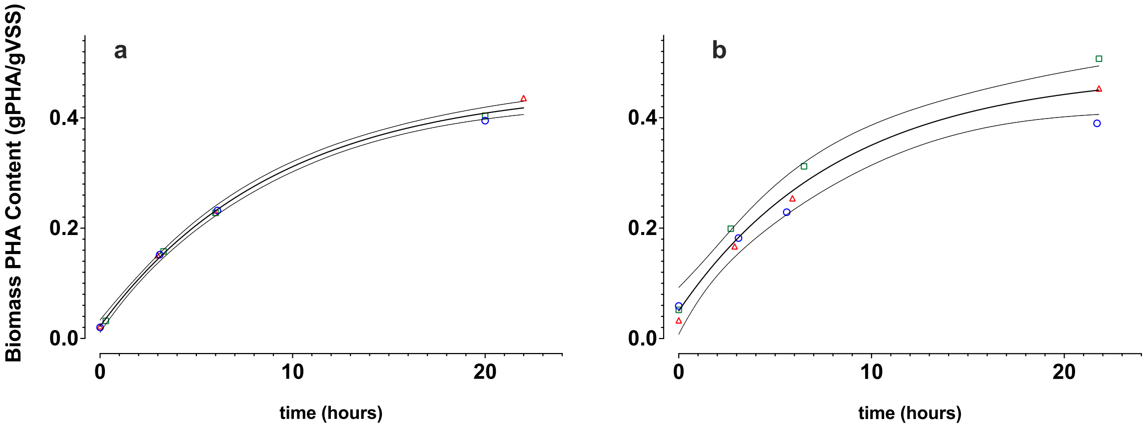

Fig. 3. Trends of biomass PHA contents for parallel pilot (a) and demo (b) production runs made in triplicate represented by blue ○, red △ green □. The average trend line was fitted by least-squares non-linear regression analysis to Eq. (1) with r^2 equal to 0.997 (pilot) and 0.962 (demo). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in PHBV, 1.67 gCOD/gPHB, and 1.92 gCOD/gPHV. Similarly, the substrate COD was estimated based on the mixture weight fraction for added acetic (1.07 gCOD/gHAc) and propionic (1.52 gCOD/gHPr) acids. Average specific production and consumption rates were calculated with respect to estimated initial active biomass levels (gCOD/gX_a/h) and from the cumulative amounts of PHA and active biomass produced, as well as substrate and oxygen consumed. Average volumetric productivity was estimated based on the mass PHA produced for the reactor working volume.

3. Results and discussion

PHA accumulation outcomes at pilot and demo scales were evaluated with respect to the bioprocess performance for PHA production and from the perspective of a potential to control polymer quality. Anticipated industrial scale process operations and practical challenges were considered from these outcomes and are discussed together with the results presented herein.

3.1. PHA production dynamics across scales

Biomass samples were similarly taken at selected times during all accumulations and this enabled monitoring of PHA production kinetics and productivity. Microscopy was undertaken to compare developments of PHA and its distribution in activated sludge flocs. It was of specific interest to assess for influences of process scale with accumulations made with the same starting WAS grab samples in replicated parallel performed pilot and demo scale production runs (Pei et al., 2023b). Fig. 2 shows qualitatively how the PHA accumulating fraction (in green) of the MLVSS increased over the accumulation period. Mixing and aeration methods were different for the pilot (mechanical mixing) and demo (aeration mixing) bioreactors. However, no significant differences were observed for biomass floc morphology and accumulated PHA distribution developments over time for the activated sludge suspended solids. The PHA accumulation distribution patterns, independent of a 20 fold change in process scale, indicate for similar intrinsic PHA accumulation dynamics for the accumulating biomass fraction of a municipal WAS microbial community given the same VFA rich feedstock using the same applied feed-on-demand control strategy.

3.2. Biomass PHA contents and yields across scales and time

Final biomass PHA content strongly influences the process economy since it impacts on productivity for the bioprocess as well as the costs in the downstream processing with recovery and purification. Effectively, downstream costs per unit PHA produced, for which drying is a significant contributor, are reduced when dried suspended solids contain a higher fraction of PHA (Bengtsson et al., 2017). Higher PHA content also means that higher extracted purity is less costly to obtain because of reduced relative amounts of non-PHA biomass that may be co-extracted (Pei et al., 2023a; Werker et al., 2023).

PHA accumulation kinetics for pilot and demo production batches were monitored from grab samples at selected time points (Fig. 3). The accumulation trends for biomass PHA content fit well to the model of first order kinetics described by Eq. (1). Biomass PHA contents for pilot versus demonstration operations similarly reached on average 0.41 and 0.45 gPHA/gVSS over 20 h, respectively (n=3) (Table 1). The kinetics and performance parameters were statistically the same ($\alpha = 0.05$) between the replicated pilot and demo scale production runs. Replication was made with distinct batches of thickened WAS, which were processed in parallel at both pilot and demo scales on the same days. In a broader context, the demo (n=3) production runs on average were robustly reproducible with reference to production runs made during PHARIO (n=52) and SCALIBUR (n=30) (Fig. 4). The experience with this WAS for *direct accumulation* spans over 5 years, including all seasons. Consistent biomass PHA content outcomes highlights this robustness for the applied bioprocess methods and reveals a stable response for PHA accumulation potential of a specific municipal WAS over several years.

In a previous study, the PHA accumulation potential was related to the biomass degree of enrichment (DE) for municipal WAS grab samples in experiments from six distinct Dutch WWTPs (Pei et al., 2022a). DE is the estimated fraction of the active biomass (X_a) that is represented by the PHA storing phenotype. The accumulation potential is the estimated plateau value for PHA content achieved during direct accumulation with an activated sludge (A_0 plus A_1 from Eq. (1)). Despite observed significant differences in activated sludge DE between the WWTPs in that previous study, an upper limit for PHA storing potential for the PHA storing phenotype in municipal activated sludge was estimated to be about 0.6 gPHA/gVSS for all 6 cases. The Bath WWTP activated sludge DE was estimated to be 55 percent (Pei et al., 2022b). Therefore, from mass balance considerations, the pilot and demo runs in the present investigation both achieved close to an expected base performance for the biomass plateau PHA content of about 0.45 gPHA/gVSS.

DE for a PHA producing MMC is well-known to be readily increased by feast-famine methods with even short term selection and enrichment involving biomass growth and turnover without net PHA storage (Valentino et al., 2013; Inoue et al., 2021). However, the established feast-famine methods for enrichment require separate bioprocess volumes or longer production cycles (Tamis et al., 2018). In contrast, improved productivity during *direct accumulation* is possible

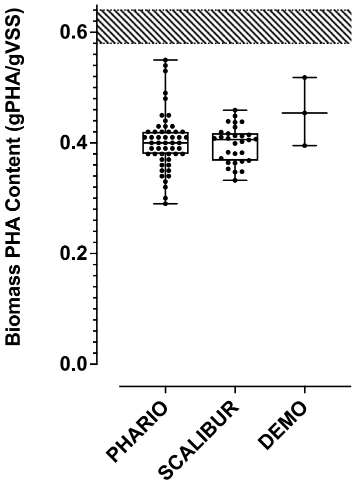

Fig. 4. Production run final biomass PHA content comparison for PHA production runs made during PHARIO (2015), SCALIBUR (2020) and demo (2020) campaigns for Bath WWTP WAS. The shaded zone gives the expected limit of PHA content for a bioprocess that stimulates selective growth during PHA accumulation as estimated by Pei et al. (2022a) and demonstrated by Estevez-Alonso et al. (2022b).

if selective growth of the PHA storing phenotype occurs concurrently with PHA storage. Such concurrent growth effectively increases the DE *in-situ* during the accumulation process. Recently, it was found that selective growth during *direct accumulation* can be reproducibly stimulated due to conditions created for interpreted reduction of metabolic ATP demand for PHA storage (Estevez-Alonso et al., 2022b). In this recent study, selective growth due to an influence of calcium resulted in three times PHA mass produced in the same accumulation time. Principles for bioprocess engineering methods to consistently stimulate the selective growth of the PHA-storing phenotypes *in-situ* during a *direct accumulation* bioprocess remain part of ongoing investigations. If this selective growth in *direct accumulation* can always be achieved with the right bioprocess conditions, it would mean that any municipal WAS could, in principle, reach a maximum PHA content of about 0.6 gPHA/gVSS (Pei et al., 2022a).

Efficiency of substrate utilization, including volatile fatty acid fermentates derived from waste sources, will become critical for up-scaled PHA production processes. A comparison of performances for the replicate parallel pilot versus demo *direct accumulation* experiments are reported in Table 1. Average PHA yield on substrate ($Y_{PHA/S}$) was statistically the same (p=0.66). Production scale was not found to influence the accumulation performance outcomes, given all other factors being kept as similar as possible (i.e. substrate quality, total accumulation time, bioprocess control methods, etc.). $Y_{PHA/S}$ outcomes in *direct accumulation* with municipal WAS (1 L laboratory scale, Pei et al., 2022a) are shown to be influenced by the length of the process. In general, the instantaneous substrate yield for PHA storage decreases over accumulation process time due, in part, to increased maintenance respiration requirements on stored PHA (De Grazia et al., 2017). Therefore, it is not surprising that reported $Y_{PHA/S}$ values for MMC PHA production, in general, cover a wide range from 0.2 to 0.7 gCOD/gCOD because different accumulation times, systems and approaches have been used (Estevez-Alonso et al., 2021a). Direct comparisons are not meaningful to make without full context because longer accumulation times will inherently have lower $Y_{PHA/S}$, with all other things being equal. PHARIO (2015) 1 m^3 pilot scale production campaigns with a similar 24 h accumulation time, the same Bath WWTP WAS source, and similar bioprocess control methods have the same context for a meaningful comparison. The PHARIO accumulations also resulted in a similar $Y_{PHA/S}$ of 0.4 ± 0.1 (n=52). Werker et al. (2020). In PHARIO, substrate quality was varied significant with fermentates derived from primary sludge, candy wastewater, as well as defined acetic/propionic acid mixtures. The cumulative experience at different scales in performance include WAS sourced from the same WWTP, with similar bioprocess methods, with different substrates, and with an interlude of many years. It suggests that municipal WWTPs could be long term and stable platforms to supply biomass with consistent outcomes in PHA production by *direct accumulation*. However, the technology would benefit by improved substrate utilization efficiency. In controlled laboratory scale experimental comparison, $Y_{PHA/S}$ was found to be higher over the same time of *direct accumulation* if bioprocess conditions stimulated the above mentioned selective growth of the PHA storing phenotype (Estevez-Alonso et al., 2022b). Conditions due to calcium for selective growth resulted in $Y_{PHA/S}$ increase from 0.2 (negative control) to 0.5 gCOD/gCOD over 48-h *direct accumulation* also using the same Bath WWTP WAS and with acetic acid as substrate. Further developments with insights and methods to achieve the above mentioned laboratory scale results of selective growth suitable for industrial scale applications are expected to significantly improve techno-economic viability of *direct accumulation* as a generically applicable PHA production supply chain approach.

There was no statistically significant influence on bioprocess performance in relative quantity of PHA produced due to an order of magnitude increase of scale. These data are summarized in (Table 1). The extent of active biomass growth was restricted, at least in part, due to the applied substrate COD:N:P composition with limiting N and P. Active biomass yields were low and not statistically significantly different (pilot versus demo pairwise t-tests, p = 0.06). Other performance parameters such as volumetric productivity (p=0.12) and substrate uptake rate (p=0.15) similarly showed no significant differences between pilot and demo parallel production runs (n=3). However, demo production runs did exhibit greater between-run variability (Fig. 3). Experiments conducted at larger scale are by their nature more difficult to control with respect to operations, protocols for sampling, and the increased practical challenges encountered including time and logistics for handling larger volumes and masses. Thus, measurement errors are expected to become intrinsically larger in moving from experiments at 200 L to 4 m^3 scales and this makes differences more difficult to determine with certainty.

Higher growth yields on average for the demo experiments reflect the corresponding higher demo scale PHA productivity on average (Table 1). Higher productivity suggests how selective growth during *direct accumulation* can positively influence process performance. The biomass PHA content trend, asymptotically approaching a steady maximum plateau level (Eq. (1)), is indicative of sustained productivity if there is sustained active growth (Valentino et al., 2015). Active growth with maintained PHA content suggests a sustained balance between carbon flux to support both active growth and PHA storage. If biomass PHA content is sustained or increasing alongside concurrent active growth then at least some of the growth is selective to the PHA storing phenotype. In this case, from mass balance, PHA storage activity still continues.

Sustained PHA contents at or approaching the plateau level alongside negligible ongoing active microbial growth is undesirable because this means the PHA accumulation process becomes increasingly inefficient due to decreasing $Y_{PHA/S}$ (Valentino et al., 2015). The yield decreases due to higher levels of catabolic substrate utilization (De Grazia et al., 2017). In practice, different types of substrates with variations in organic compositions and nutrients may be expected to elicit varied responses in storage and growth, making a pre-selected accumulation termination time an inadequate operations strategy. For maximized production and substrate utilization efficiency from batch-to-batch, longer or shorter process times may be required. As a result, online methods to monitor and interpret the accumulation developments in real-time including following trends for ongoing (selective) growth and PHA storage activities are expected to become essential for industrial-scale operations. Infrared spectroscopy and respiration monitoring methods have been proposed (Werker et al., 2014; Cruz et al., 2015; Werker et al., 2022).

Table 1
Comparison of performance in three replicate parallel pilot and demo scale PHA production experiments.

	PHA yield gCOD/gCOD	X yield gCOD/gCOD	O_2 yield gCOD/gCOD	PHA content gPHA/gVSS	Uptake rate mgCOD/g X/h	Specific PHA productivity mgPHA/gX/h	Volumetric productivity mgPHA/L/h
Pilot 1	0.43	0.07	0.30	0.44	165	41	98
Pilot 2	0.43	0.07	0.31	0.40	146	36	101
Pilot 3	0.39	0.06	0.32	0.39	154	34	94
Demo 1	0.46	0.15	0.40	0.45	174	56	138
Demo 2	0.42	0.11	0.29	0.51	231	72	246
Demo 3	0.30	0.17	0.37	0.39	219	49	157
Pilot Average	0.42 ± 0.02	0.07 ± 0.00	0.31 ± 0.01	0.41 ± 0.02	155 ± 8	37 ± 3	98 ± 3
Demo Average	0.39 ± 0.07	0.14 ± 0.02	0.35 ± 0.05	0.45 ± 0.05	208 ± 25	59 ± 10	180 ± 47

3.3. Quality of the produced PHBV

Each production batch at demo 4 m^3 scale yielded in the order of 10–20 kg PHBV. These PHBVs were subsequently recovered also at demonstration scale and property specifications have been evaluated on selected batches and reported elsewhere (Molenveld et al., 2022). The demonstration scale polymer recovery is beyond the scope of the present investigation. However, critical assessments were made to address questions of up-scaled production quality and quality control with insights from comparisons made between properties of PHARIO, SCALIBUR, and demo scale production batches (Werker et al., 2020).

Commercial production methods require to ensure consistent (predictable) outcomes in monomer composition, molecular weight (M_w), and polymer crystallization behaviour. Mixed and pure cultures alike inherently produce PHBV co-polymer blends that may be readily fractionated into sub-blend components that are distinct in average molecular weight and/or average monomer composition (Wang et al., 2001; Laycock et al., 2014; Werker et al., 2020). The blend components are separable due to differences in solubility as a function of blend fraction average molecular weight and average co-monomer content. Blend components may respectively be more or less crystalline in isolation as may be expected due to respective fraction differences in average monomer composition (Laycock et al., 2013). The substrate composition, including VFA and non-VFA COD, used in mixed microbial culture PHA production is shown to systematically determine average monomer composition of the accumulated co-polymer blends (Gurieff and Lant, 2007; Laycock et al., 2013; Werker et al., 2020).

Random co-polymers inherently with average M_w of up to about 1500 kD and a polydispersity (PDI) index close to 2 are expected when a substrate rich in VFAs is fed consequently to WAS in an accumulation process (Estevez-Alonso et al., 2022a; Werker et al., 2020). Replicate standardized test tube extractions were performed on dried biomass grab samples from PHARIO (n=13), SCALIBUR (n=25) and demo (n=6) production batches. Note that the dried PHARIO PHA-rich biomass granulate samples had been produced during 2015 and stored dried for more than 5 years. Mass balances from these standard extractions gave statistically similar efficiencies (Fig. 5) and suggested that essentially all the PHBV in the biomass is extractable. In practice, some residual solvent containing dissolved PHA may be expected to remain with the spent biomass particulate matter. Therefore, while these polymers are readily extractable, not all the polymer may be practically recoverable at industrial scale.

Differences in the extracted PHBV molecular weight are correlated directly with measurements of intrinsic viscosity (Fig. 6 b) based on the Mark–Houwink equation using representative equation constants (Werker et al., 2023). PHA production with consistently controlled (higher) molecular weight (intrinsic viscosity) is desired because downstream melt processing for compounding and conversion will cause molecular weight losses. Molecular weights above about 250 kDa are required to retain mechanical properties (Luo et al., 2002). To minimize molecular weight loss, it is necessary to reduce decomposition rates during extraction at elevated temperatures. Therefore, the thermal decomposition temperature (T_d) of the polymers should be maximized after an accumulation bioprocess (Werker et al., 2013, 2020). Acidification leads to a significant increase in the T_d of the PHBV, even while it still remains part of the biomass (Chen et al., 2017; Pei et al., 2023a). T_d was consistently high for PHARIO, SCALIBUR and demo dried biomass batches (Fig. 6). However, M_w quality was variable during PHARIO. PHARIO M_w variability was attributed, in part, to conditions of the biomass drying (Werker et al., 2020). In the PHARIO project, the PHA-rich biomass was dried at 70 °C, whereas lab-scale experiments during PHARIO, and confirmed more recently, support that rapid initial heating during drying results in recovered PHAs with consistently higher molecular weights (Estevez-Alonso et al., 2021a). The effect of rapid heating is attributed to the reduced duration of temperature-dependent enzyme activity before the thermal deactivation of depolymerases (Pei et al., 2023a). The SCALIBUR and demo production runs downstream processing steps of dewatering and drying were conducted in a manner similar to the PHARIO project, with the key difference being the use of a higher initial drying temperature (120 °C for 30 min or flash drying at higher than 100 °C). This adjustment resulted in reduced product molecular weight variation with consistent higher values compared to the PHARIO batches (Fig. 6). The demo accumulations, in applying rapid flash heating to 100 °C via a commercial drum drier, supports that up-scaled production with conserved reproducible (higher) molecular weight quality can be readily achieved.

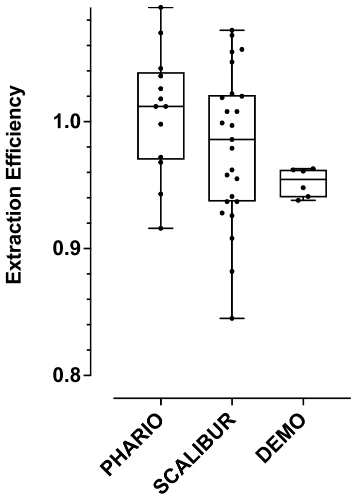

Fig. 5. PHA extraction efficiency (grams PHA dissolved into the solvent over grams PHA contained in the biomass added to the solvent for the extraction) from dried PHA rich biomass obtained from PHARIO (2015), SCALIBUR (2020) and Demo (2020) production run grab samples.

The average monomer composition and co-polymer blend distribution affects the PHBV properties including melting and crystallization temperature ranges and rates. These properties influence thermal processing conditions and cycle times (processability) towards yielding a hardened polymer microstructure (property specifications) critical for PHA applications. PHARIO project demonstrated that the average

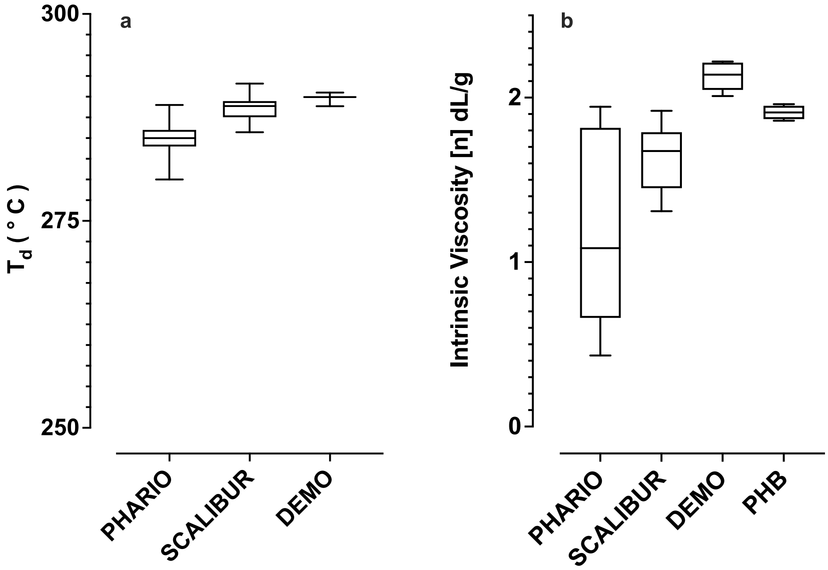

Fig. 6. Average outcomes from analyses of decomposition temperatures (T_d) by TGA and average molecular weights (M_w) represented by intrinsic viscosities for PHARIO, SCALIBUR and demo production runs. Intrinsic viscosity for replicate measurements of a commercial (BIOMER) PHB are shown for reference of method reproducibility and accepted commercial quality.

3HV content of PHBV was predictable from the feedstock composition comprising VFA and non-VFA dissolved organic carbon (non-VFA DOC) (Werker et al., 2020). Primary sludge fermentate resulted in co-polymer blends with 33 ± 3 weight percent average 3HV content. Variability of 3HV content in PHARIO was due, in part, to batch-to-batch differences in non-VFA DOC of the fermentate. The non-VFA DOC has previously been shown to contribute to 3HB synthesis (Gurieff, 2007). Inclusion of waste derived fermentate supply was beyond the scope for the efforts of both SCALIBUR and demo PHA production activities. It was nevertheless an objective in SCALIBUR and demo projects to use a defined substrate mixture of acetic and propionic acids to produce a targeted PHBV having a similar average 3HV content compared to a PHBV produced with primary sludge fermentate. Multi-linear regression least squares analysis based on feedstock composition confirmed an ability to produce PHBVs with predictable co-monomer compositions (Fig. 7). Predictability is assumed to be possible given the same biomass source coupled to the same methods and conditions applied for the PHA production process. SCALIBUR and demo production runs supported a possibility to systematically tune mixed culture average monomer compositions by adjusting feedstock VFA concentrations. Distinctly different feedstocks can be used to produce PHAs with similar average monomer contents.

3.4. Monomer composition, co-polymer blends, and crystallization

Even if average monomer composition is predictable from substrate composition, average 3HV content in PHBV is insufficient to describe polymer properties. Consideration of monomer composition alone misses an independent influence of the co-polymer blend distribution on polymer melt and crystallization behaviour. Distinct substrates (acetic:propionic acid mixtures, or waste fermentates) resulted in PHBVs with predictably similar weight average 3HV contents, but the recovered polymers had distinctly different co-polymer blend compositions. These differences are shown by DSC. Fig. 8a shows typical outcomes of the polymer glass transition, cold crystallization, and melting in the second DSC heating ramp for PHARIO, SCALIBUR and demo extracted PHBVs from respective production batches. All had nominally 36 average weight percent 3HV content. SCALIBUR and demo PHBVs (made on acetic:propionic acid mixtures) were similar. PHARIO PHBV (made on a waste fermentate from municipal primary sludge) was distinctly different. The PHARIO PHBV did not crystallize during quenching after the first heating ramp, nor did it show measurable peaks of exothermic cold crystallization or endothermic melting. (Fig. 9a).

PHBV co-polymers undergo secondary crystallization when aged at room temperature (Fig. 8b). Both SCALIBUR and PHARIO PHBVs crystallized with ageing as shown by the heating ramp after ageing and, compared to Fig. 8a for the same sample with no ageing. The developed broad melting peak starting at about 46 °C. However, the aged polymer median melt temperature was 93 for the PHARIO PHBV versus 153 °C for the SCALIBUR. The aged PHARIO PHBV was also less crystalline than the SCALIBUR ($\Delta\ H_m$ of 39 versus 60 J/gPHA, respectively). The PHARIO versus SCALIBUR/demo PHBVs represent completely different co-polymer blends notwithstanding having the same average 3HV contents. The PHA research literature gives principal focus on outcomes of polymer type defined by just average monomer content (Verhoogt et al., 1994; Anjum et al., 2016; Ganesh Saratale et al., 2021). The crystallization rate and microstructure that develops in time as the outcome of cooling after melt processing is coupled directly to processability and the hardened article mechanical properties. It determines the material scope and limitations for and in applications. The study results emphasize that upscaled quality control for MMC PHBV production is not so much about controlling PHBV average monomer composition. It is about making co-polymer blends of PHBVs that crystallize consistently from one batch to the next in the production of thermoplastic materials for a given application.

Rapidly quenching a melted PHA to below the glass transition will produce micro-structures comprising regions of mobile amorphous, rigid amorphous, and crystalline microstructures in these semi-crystalline co-polymer blends (Di Lorenzo and Righetti, 2013). Cold crystallization relates to exothermic crystal growth of the rigid amorphous fraction. The polymer melt enthalpy is due to contributions of crystalline phases formed during quenching and during cold crystallization. SCALIBUR and demo PHBV had higher cold crystallization enthalpy but lower melt enthalpy compared to values for pure PHB estimated at $\Delta\ H_{cc}$ = 14 J/gPHA and $\Delta\ H_m$ = 94 J/gPHA). The higher cold crystallization enthalpy compared to PHB suggests greater complexity of microstructure at the interface between crystalline and amorphous phases. The role of this complexity on the material properties for applications is part of ongoing investigation.

Glass transition temperatures (Fig. 10) approximately followed expectations from PHARIO of an influence of average 3HV content.

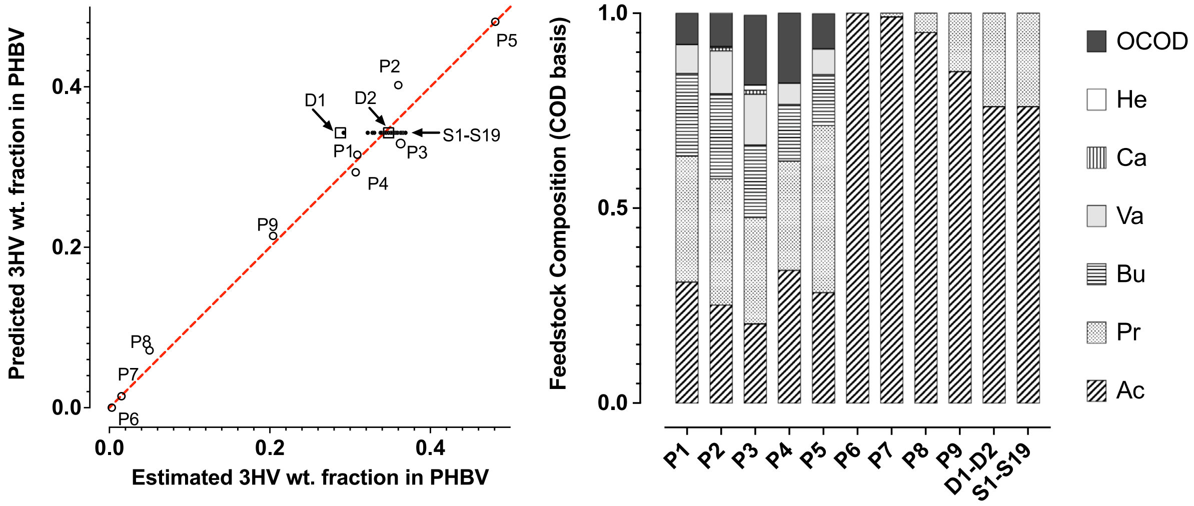

Fig. 7. Measured versus predicted 3-hydroxyvalerate (3HV) weight fraction in PHBV as a function of PHBVs produced using a range of respective feedstocks with varying acetate (Ac), propionate (Pr), butyrate (Bu), valerate (Va), caproate (Ca), heptanoate (He), and other COD contents (OCOD) during PHARIO (P), SCALIBUR (S), and demo (D) production campaigns. 3HV model prediction was by multilinear least squares analysis as a function of the feedstock COD composition.

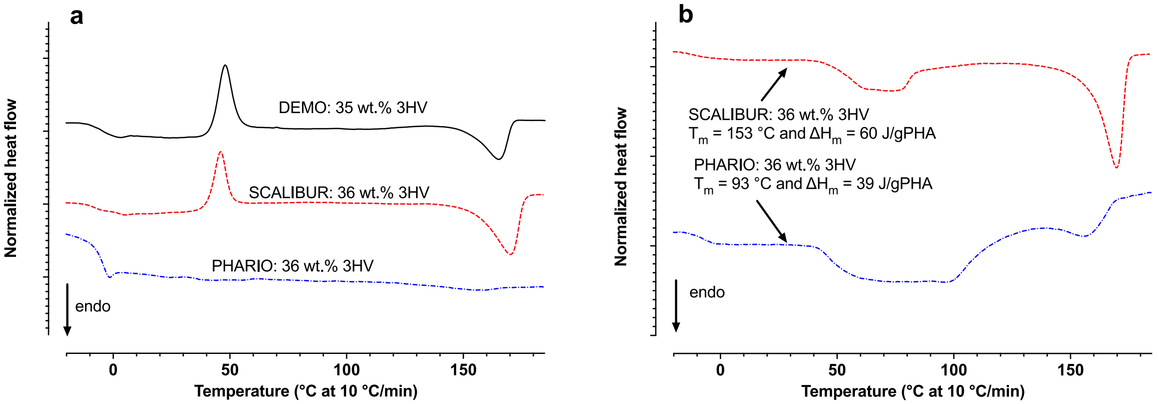

Fig. 8. Normalized DSC heat flow during heating ramps on PHBVs from PHARIO, SCALIBUR, and demo production runs: (a) directly after melting and quenching the polymers and (b) after ageing the melt and quenched polymers at room temperature for 1 week. Heat flows reveal initially a base-line shift indicative of a glass transition, distinctive positive peaks of cold crystallization, and negative peaks revealing crystal melting temperature ranges.

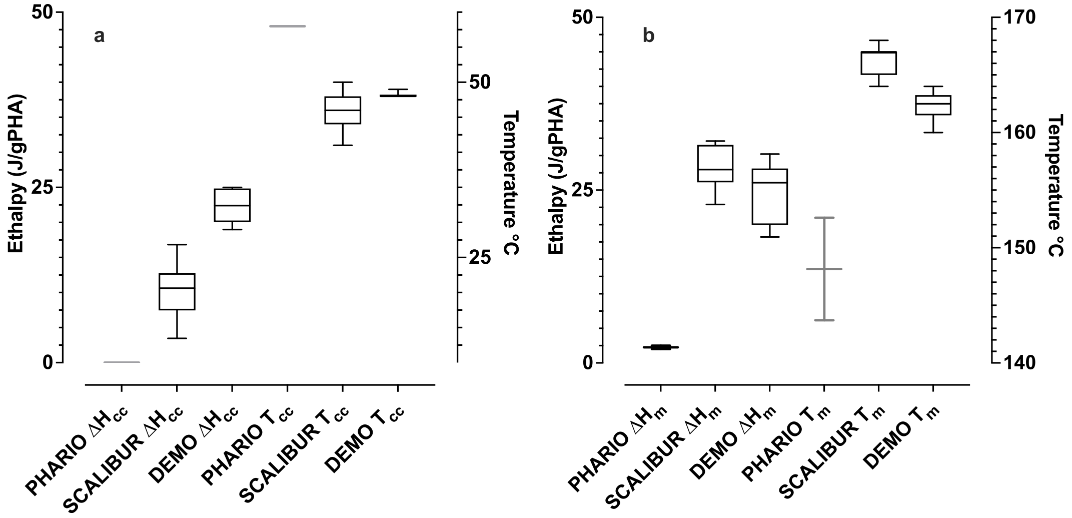

Fig. 9. With reference to Fig. 8a, estimated average enthalpies and temperatures for (a) cold crystallization, and (b) melting peaks for extracted polymers from n replicate PHARIO (n=2, 3HV = 34 wt. percent), SCALIBUR (n=19, 3HV = 35±2 wt. percent) and demo (n=2, 3HV = 32 wt. percent with extractions in triplicate) samples. All samples had between 30 and 35 weight percent 3HV in PHBV.

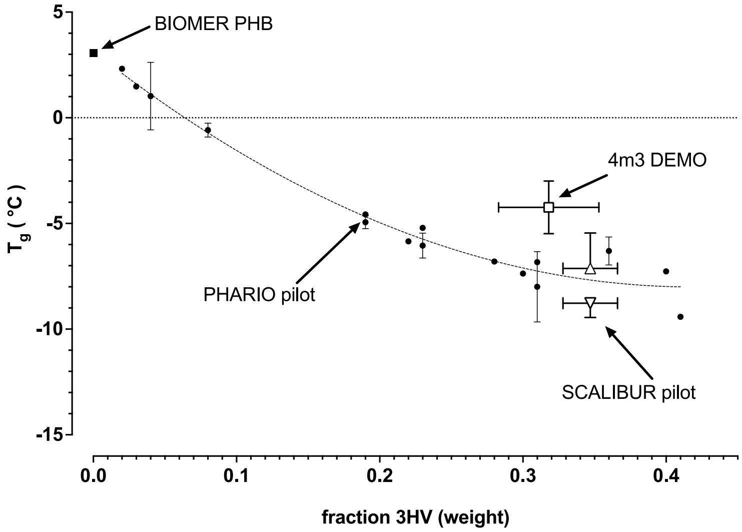

Fig. 10. Estimated glass transition temperature (Tg) as a function of PHBV composition expressed as 3HV weight fraction for PHARIO (•), SCALIBUR (△), and demo production runs (□). Tg for commercial (BIOMER) PHB (■) also shown for reference.

However, here again deviations from the trend are interpreted to be due to the nature of the co-polymer blend composition. SCALIBUR PHBVs exhibited two glass temperatures (T_g). Two T_g values suggest that the extracted SCALIBUR PHBV comprises an immiscible blend. PHBV co-polymer blends are likely to be immiscible when the main blend fractions are sufficiently different in average 3HV content by about 11 wt%. Werker et al. (2020).

The PHBV research literature has stressed with interest over many years to produce less inherently crystalline (brittle) PHAs than PHB by introducing co-monomers like 3-hydroxyvalerate into the polymer chains (Savenkova et al., 2000). However, less crystalline PHBVs like those produced during PHARIO crystallize very slowly over hours and days (Werker et al., 2023). Slow crystallization rate is a counter-challenge in processability for PHBVs as a trade-off to the challenges of brittleness for PHB for applications. Interestingly, the SCALIBUR and demo PHBVs had reduced crystallinity compared to PHB but hardened more quickly than PHARIO PHBV due to an interpreted significant more crystalline blend fraction. Blending as a means to improve both processability and application property specifications as an element of PHBV industrial production quality control needs further attention and development. Monomer composition and co-polymer blend engineering includes the bioprocess step defined by the VFA-rich substrate, biomass and PHA accumulation methods. Further blending for engineering crystallization properties and for absorbing batch-to-batch production variability may include mixing batches of inherently slower and quicker crystallizing blend fractions in the steps of extraction and purification (Verhoogt et al., 1994; Werker et al., 2020).

The batch-to-batch polymer properties in replicate accumulation experiments demonstrated stability of productivity and quality for both pilot versus demo scale production runs. This reproducibility supports that even municipal WAS is applicable in technical feasibility for up-scaled production process quality control with respect to making polymers that are effectively similar for a given feedstock and for a given bioprocess production methodology. The overall results of promise for quality control (average molecular weight, polymer average monomer composition, and crystallization/melt kinetics) underscore a suitability for supply chains including municipal WAS as a raw material for direct use in industrial scale PHA production.

3.5. *Up-scaling and practical considerations*

The demo scale work supports technology readiness for PHA production by *direct accumulation* using municipal WAS. It affirms a technology readiness level (TRL) of 7 - “system prototype demonstration in operational environment”. Up-scaling to 4 m^3 was instructive to address practical aspects of process and materials management challenges that are typically out of the scope of lab and even pilot scale experiments. Three selected challenges were reviewed including, (1) bioprocess operations (temperature, aeration and foaming) (2) downstream processing, and (3) production quality control coupled to feedstocks and production volumes necessary for future industrial scale PHA supply chains.

3.5.1. *Bioprocess challenges*

Temperature influences both microbial metabolic activity and oxygen solubility. Municipal WAS acclimates to seasonal waste water temperatures with an annual range from about 12 to 26 °C for northern climates (Werker, 2006). Activated sludge biomass is shown to accommodate accumulation temperatures in this range even with sudden changes of temperature between 15 and 30 °C (De Grazia et al., 2017). The demo scale runs were conducted during November and December with a non-insulated vessel. Starting temperatures were brought to 22 °C initially with active heating from as low as 10 °C. Process aerobic catabolic activity was sufficient to drive temperatures further to 28.5 °C (see: Table 2). However, with increased temperature, the process aeration capacity became challenged due to the decrease in oxygen solubility with respect to the levels of oxygen demand proportional to active biomass MLVSS concentrations and the associated levels of metabolic activity (see: Table 2). Higher starting MLVSS levels are desired for maximal volumetric productivity. Lower oxygen levels are advantageous as a means to limit oxygen demand due to nitrifying microorganisms in WAS and to exploit nitrate production without loss in PHA production rates when ammonia levels in the feedstock are significant (Estevez-Alonso et al., 2021b). However, stringent oxygen limitation will reduce productivity due to slower accumulation kinetics (Pratt et al., 2019). The accumulation bioprocess design must accommodate oxygen demand, dissolved oxygen control, and system biomass loading for the range of possible operating temperatures and methods of temperature regulation.

Aeration, mixing and presence of surfactants and/or hydrophobic cells may promote foaming in activated sludge (Petrovski et al., 2011). As foams are generated and rise above the working volume into the reactor headspace, they inherently drain and become unstable. However, foams also become stabilized by surfactants like proteins and foam drainage can also be impeded by the entrained mixed liquor suspended solids. Feedstocks will need to be evaluated for components that may promote foam generation, and/or foam stability. In

Table 2
Comparison of the operational parameters between the parallel pilot and demo operations.

	X_{start} gVSS/L	X_{end} gVSS/L	Duration h	T_{start} °C	T_{end} °C	DO_{start} mgO$_2$/L	DO_{min} mgO$_2$/L	pH_{min}	pH_{max}
Pilot 1	2.4	2.9	22	20	20	9.5	2.9	7.0	8.6
Pilot 2	2.8	3.1	20	20	20	9.3	2.6	6.7	8.6
Pilot 3	2.8	3.0	20	20	20	9.1	2.5	6.9	8.5
Demo 1	2.6	3.7	22	20	24	11.0	1.0	7.0	8.9
Demo 2	4.1	5.1	22	22	29	7.6	0.2	7.2	8.6
Demo 3	3.5	5.5	22	25	33	9.2	0	6.9	8.5
Pilot Average	2.7 ± 0.2	3.0 ± 0.1	–	20 ± 0	20 ± 0	9.3 ± 0.2	2.7 ± 0.2	6.9 ± 0.1	8.6 ± 0.0
Demo Average	3.4 ± 0.6	4.8 ± 0.8	–	22 ± 2	29 ± 4	9.3 ± 1.4	0.4 ± 0.4	7.0 ± 0.1	8.7 ± 0.2

the present investigation, a recirculation flow with spray nozzle was included in the design to dampen foam production. Antifoaming agents were not used in these experiments to avoid decrease in oxygen mass transfer rates and to be able to evaluate the process performance with as little added complexity as possible (McClure et al., 2017). However, a simple spray for foam control was not sufficient in at least some of the demo production runs when foaming was excessive and could not be contained in the reactor headspace. The reactor geometry, free headspace volume and, if necessary, antifoam mechanical or chemical control methods will need to be integral to an upscaled bioprocess design.

3.5.2. Downstream process challenges

Downstream processing remains a bottleneck for MMC PHA production (Estevez-Alonso et al., 2021a). Methods of PHA recovery have been reviewed extensively over the past 10 years (Mannina et al., 2020; Pagliano et al., 2021). However, the research literature does not generally recognize that downstream processing for maintained polymer quality control starts immediately after the termination of the accumulation bioprocess and includes all aspects of unit processes, environments and time scales of the PHA-rich biomass handling during and between steps of polymer preservation (acidification, dewatering, drying) and recovery (blending, extraction, and purification). Steps and time scales are important for preserving the *in-vivo* polymer quantity and quality starting from the time of bioprocess termination with end of aeration (Pei et al., 2023a). The present work supports, for examples, that achieving a high thermal stability, pH related depolymerase rate inhibition, and rapid temperature increase for drying, are all critical aspects in downstream processing for molecular weight quality preservation.

3.5.3. Industrial supply chain challenges

Supply chains of biomass and VFA-rich feedstock for MMC PHA production should come up to at least 5 to 50 kilotons recovered PHA/year to be relevant for the biopolymer market (Bauchmüller et al., 2021). The bottleneck for PHA recovery is due to investment cost of recovery. A good scale of economy for an extraction process is anticipated to be at least 5 kilotons per year. Dried PHA-rich biomass exhibits a long shelf-life. Therefore, it is an undue constraint to assume that every location of PHA accumulation (i.e. the bioprocess) must be accompanied by its own PHA recovery process. Since individual PHA accumulation facilities will likely be limited in capacity to the order of magnitude of 1 to 2 kiloton PHA/year, then it makes sense to decouple the business model of the step of production of a dried PHA-rich biomass from the downstream steps of PHA recovery (Werker et al., 2020). Batches of dried PHA-rich biomass are already semi-products that can be supplied, sold, and masterbatched. Thus, batches of dried PHA-rich biomass can become well-defined traded products supplying recovery facilities that maintain a good economy of scale and can ensure blending of batches to obtain grades of PHA of consistent quality (i.e. melt and crystallization behaviour).

Direct accumulation of PHA with municipal WAS creates a generic and distributed global potential to produce PHAs within the context of larger urban centre organic waste management activities. Waste activated sludge is ubiquitously produced and undervalued for its potentially significant capacity to produce PHA. Lab through to demo scale research demonstrates feasibility of utilizing all sorts of VFA-rich feedstocks sourced from various industries in addition to municipal primary sludge fermentate (Estevez-Alonso et al., 2021a). Sourcing VFA for supply chains is not trivial even if many kinds of wastes are shown to be exploitable. Even low concentrations of VFAs in a fermentate are suitable for the PHA production bioprocess so long as a sufficient VFA mass can be supplied (Werker et al., 2022). In practice, it will not likely make sense to transport large volumes of dilute (0.5 to 50 gCOD/L) feedstocks for VFA supply in *direct accumulation* PHA production. Instead, thickened WAS can be transported for *direct accumulation* to locations with significant readily available organic residuals as sources for VFAs. The logistics, costs and the regional scenarios for locations of sites to combine WAS with VFAs to produce PHAs will need cooperative strategic planning such that a meaningful critical mass of PHA is produced that can serve socio-economic bioplastic needs of regional stakeholders (Bauchmuller et al., 2021). Approaches to maximize collective economic benefit with reduced environmental footprint are analogous to considerations of logistics in sludge transport and its management to selected regional sites for anaerobic digestion (Henriques et al., 2020). There are other analogous and positive outcomes tested in viable scenarios of converting renewable resources from municipal organic waste management services for PHA supply (Moretto et al., 2020). In practice a mix of approaches and supply chains will be necessary to satisfy, at a regional level, the collective overall global demand for biopolymers.

4. Conclusions

The present investigation supports, with reference to the literature, a consistency of experiences from 1 L laboratory to 4 m^3 demo bioprocess volumes when producing PHBV co-polymer blends by *direct accumulation* using municipal WAS as the biomass source. Similarly applied feed-on-demand methods resulted in comparable performances for quantity and quality of the PHBVs produced independent of the production scale. Selective growth of the PHA storing phenotype can ensue during *direct accumulation* and this growth improves volumetric productivity. Industrial scale production of PHBVs requires quality control of average co-monomer content, molecular weight, and co-polymer blend composition. Average monomer content and co-polymer blend compositions were found to be robust and predictable but feedstock quality dependent. The co-polymer blend composition can include blend fractions with distinctly different respective blend fraction crystallization behaviours. Blend engineering in a centralized PHA extraction facility is anticipated to become an important part of industrial scale MMC PHA production quality control and, ultimately, renewable resource value generation.

CRediT authorship contribution statement

Ruizhe Pei: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Erik de Vries:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **Angel Estévez:** Writing – review & editing, Project administration, Methodology, Investigation. **João Sousa:** Project administration, Investigation, Formal analysis. **Henk Dijkman:** Writing – review & editing, Investigation, Formal analysis. **Jelmer Tamis:** Investigation, Formal analysis. **Alan Werker:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

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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Data availability

Data will be made available on request.

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