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Thermal pre-processing before extraction of polyhydroxyalkanoates for molecular weight quality control

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ABSTRACT

Past studies have repeatedly demonstrated the technical feasibility to produce polyhydroxyalkanoate (PHA) using bacterial biomass of mixed microbial cultures (MMCs). Commercial quality grades of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHBV, can be produced with control of average monomer composition. However, demonstration of PHBV production and recovery with quality control of molecular weight (MW) distribution has been lacking in the research literature. Towards this goal, a workflow has been developed for characterizing molecular weight control by thermal treatment pre-processing of dried PHA-rich biomass before solvent extraction. Dimethyl carbonate (DMC) was a suitable solvent in this workflow in the routine evaluation of extractable PHA. From assessments of DMC extraction using differential scanning calorimetry, 125 °C was selected for nominally 100 percent extraction yield independent of polymer 3-hydroxyvalerate (3HV) content (2 to 41 wt.% 3HV) and molecular weight (100 to 1400 kDa). Intrinsic viscosity measurements of PHBV in DMC at 60 °C was used for molecular weight monitoring. Mark-Houwink constants, α (0.738 ± 0.010) and LogK (-2.016 ± 0.025), were estimated for a PHBV co-polymer blend having 36 wt.% 3HV. A model of random scission supported that weight average molecular weight (M_w) was a more robust metric, compared to number average molecular weight (M_n), for assessing the polymer scission rates. During isothermal heat treatment for a given biomass batch, interpreted scission rate was reproducible and commonly, but not always, constant in time. Scission rates between biomass batches were also variable. Measured properties of the polymer in the biomass (thermal stability, biomass PHA content, PHBV grade, initial moisture content) could not be correlated to this observed batch-to-batch variation of scission rate. Molecular weight loss before extraction did not influence the melting temperatures of the co-polymer blends of PHBV evaluated over a wide sub-eutectic range of average 3HV content. Molecular weight changes for these PHBV co-polymer blends were considered to have likely influenced the nature of blend 3HV distribution, and consequently, crystallization behaviour. Molecular weight loss effects on crystallization behaviour at constant PHBV average 3HV wt.% content could then have contributed to the observed variability for glass transition temperatures and melting enthalpies. However, a reproducible correlation between this variability and MW change was not observed.

Abbreviations: α, K, Mark-Houwink constants; [η], Intrinsic viscosity; ΔH_m. ΔH_{gel}, Melting enthalpy and gelation enthalpy; BOH, 2-butanol; DMC, dimethyl carbonate; DSC, differential scanning calorimetry; 3HV, 3-hydroxyvalerate; MH, Mark-Houwink; MMC, Mixed microbial culture; M_n, M_w, number and weight average molecular weights; MW, MWD, Molecular weight and molecular weight distribution; n_{sn}, n_{sw}, Number and weight average scission numbers; PHB, poly(3-hydroxybutyrate); PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PDI or Đ, Polydispersity index; r_{sn}, r_{sw}, Number and weight average scission rates; T_d, polymer decomposition temperature (at 10 °C/min); T_{50}, T_g, Median melting temperature and glass transition temperature; TGA, thermogravimetric analysis; TS, (dried) total solids; VS, (dried) volatile solids.

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1. Introduction

Polyhydroxyalkanoates (PHAs) are a class of biobased polyesters. They are accumulated as intracellular granules by many species of naturally occurring bacteria [1]. Polymer granules will accumulate in these bacteria due to a growth limitation while an exogenous organic substrate becomes otherwise transiently available. PHAs can be purified from biomass [2,3] and these semi-crystalline polymers have commercial interest because of a range of achievable thermoplastic and mechanical properties [4]. They are furthermore completely biodegradable in microbially active environments [5,6].

Research developments to motivate supply chains for wider commercial production capacity of PHAs often explore methods for anticipated upstream cost reduction in polymer production [7,8]. One strategy to reduce upstream costs is to use wastes as substrates for pure culture production methods [9,10]. Further cost reductions may be achieved by open culture production methods thereby avoiding a need for sterilization with axenic bioprocesses. Technology readiness for open (mixed) microbial culture (MMC) PHA production has been repeatedly demonstrated [11]. MMC biomass with significant PHA storing capacity can be realized as an integral part of municipal wastewater treatment [12]. Waste municipal activated sludge is ubiquitously available and may be estimated to roughly represent an annual production capacity of 14 000 tons PHA per million-person equivalent discharging to municipal wastewater treatment plants (based on 30 g-activated sludge dry solids produced per person equivalent per year and recent research results - [13,14]). Supply chains of this order in magnitude can serve several interesting niche applications where well-timed biodegradation of plastic articles, or wear debris therefrom, is needed [15]. However, production capacity in quantity does not solve the all-to-often unspoken challenge of article application specific demands on the bioplastic production quality control.

Controlled reproducible physical and chemical properties are necessary towards demands in property specifications in processing methods and in targeted applications [16]. There are many different types of PHAs, and these types are broadly reviewed with respect to the average monomer composition [17]. Mixed microbial cultures have most commonly been used to produce poly (3-hydroxybutyrate-co-3-hydroxyvalerate) or PHBVs. PHBVs are derived from feeding an accumulating bacterial biomass with volatile acid rich substrates. Such PHBVs are co-polymer blends with respect to monomer and molecular weight (MW) distributions [18]. Many kinds of applications are anticipated for PHBVs, but the available grades of PHA based bioplastics in general are limited or sometimes uncertain [19]. Investments in application developments require stable supplies of specific and reproducible PHA grades with well-defined polymer property specifications. Such specifications are tied to both the monomer composition and its distribution, as well as the MW distribution [16].

Average monomer composition and its distribution in PHBVs influences crystallization and crystallinity which will strongly influence the polymer chemomechanical properties [4]. Monomer average compositions were shown to be robustly predictable from the feedstock composition [18]. Therefore, even the direct accumulation of PHA in full scale surplus municipal waste activated sludge can result in reproducible outcomes of polymer average monomer composition. It was further shown that co-polymer blend quality control can be achieved in the downstream processing, by selective blending of PHA-rich biomass production batches before steps of polymer extraction and purification.

MW distribution influences end-use properties of biopolymers, via resulting crystallization rates and the outcome of macromolecular and supra- macromolecular structures [4]. Higher average MWs can challenge extraction processes due to increased solvent viscosity for the same extracted polymer concentration [20]. Lack of control in details for the accumulation process and/or downstream processing will result in unpredictably variable MW distributions that can be too low, or even too high, for intended production processing methods or applications [18]. Research developments towards systematic methods in molecular weight control for MMC PHA production are lacking in the research literature.

One approach for MW control can start with process methods that deliver reproducible maximal molecular weight distribution in PHA granules during an MMC accumulation process. Subsequent downstream processing steps in the polymer purification will tend to reduce molecular weight distribution, to a greater or lesser extent, depending on the recovery methods and conditions [21]. A goal is to establish quality control by engineering to smooth out input MW variability and to adapt to predictable effects on MW quality from conditions applied in the steps of polymer purification. All steps combined should permit to deliver a recovered PHA with defined MW distribution and average molecular mass [22]. In recent work, methods to produce MMC PHAs with weight average molecular mass of about 1500 kDa on average, and up to 2000 kDa, were validated [20]. Molecular weights of 1500 kDa are significantly higher on average than have been typically reported (circa 100 to 1000 kDa) for mixed culture PHA production [18,23–29]. In the present work, it was of interest to understand if thermal pre-treatment could be applied to predictably decrease MW for the polymer in the biomass to a targeted level before next steps of extraction and purification.

Thermal pre-treatment results in MW loss of PHA is due to random scission of ester groups at a rate dependant on temperature following an Arrhenius relationship [30–33]. In the present work, the kinetics and predictability of MW loss due to an isothermal thermal treatment of PHBV-rich biomass before solvent extraction was evaluated. Selected subsamples from batches of oven dried PHBV-rich-biomass, representing a range from 2 to 41 wt. percent of average 3-hydroxyvalerate in PHBV, were subject to a defined heat treatment time in air at 180 °C. The PHBV was extracted through a solvent extraction method with dimethyl carbonate that was tuned as part of the study. Resulting kinetics in molecular weight loss were monitored together with any evidence for molecular weight dependant effects on extraction yields, total extract purity, or on the degraded polymer thermal properties. A model of random scission was developed and tested to be able to interpret molecular weight loss trends measured by solution rheology. Inherently, from these evaluations a workflow with methods of standard laboratory extraction, mass balances, and polymer assessments were established and validated. Insights on the random scission process, and on steps for engineering quality control of MMC PHA molecular weight during the polymer recovery, have been advanced and are presented herein.

2. Methods and materials

2.1. PHA-rich-biomass, extraction, and polymer solvent solutions

Dried PHA-rich-biomass grab samples were produced as part of the PHARIO project [18]. In PHARIO, municipal activated sludge was accumulated with poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or PHBV, by fed-batch feed-on-demand methods at pilot scale over 24 h. On average ($n = 59$) 0.40±0.05 gPHA/gVS was accumulated per batch at pilot scale. The post accumulation suspended solids of PHA-rich biomass was acidified to pH 2 with sulphuric acid to increase the polymer thermal decomposition temperature prior to dewatering and oven drying, as previously presented [34]. Dried biomass solids with nominally 3% residual moisture content were ground and sieved. A granulate with less than 2 mm maximum particle size was collected and stored at room temperature in sealed 1 L Nalgene bottles. For the present work, selected batches of this dried, granulated, and stored PHA-rich-biomass were used. The selected batches covered a range in grades of PHBV (2 to 41 wt.% 3-hydroxyvalerate) and weight average molecular mass (100 to 1400 kDa). PHA quantity and quality in the dried biomass were assessed by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and pyrolysis with gas chromatography combined with mass spectroscopy (PyGCMS) (see analytical

methods).

Prior to extraction, PHA-rich biomass samples were re-dried at 105 °C for 30 min. In thermal treatment experiments, a set of subsamples were obtained after subjecting the subsamples to an incubation in air at 180 °C from 0 up to 120 min. The PHA was extracted from the subsamples in 10 mL dimethyl carbonate (DMC, Sigma-Aldrich Reagent-Plus®, 99%). Extractions were performed in tare weighed 20 mm diameter glass digestion tubes (Hach, LZP065). Weighed amounts of PHA-rich biomass granulate were added to the tubes to achieve a maximum theoretical extraction polymer concentration, the "extraction loading", of either 20 or 50 mgPHA/mL. A weighed mass of DMC was added according to the target extraction loading, and tubes were sealed with respective tare weighed caps. Tube contents were vortex mixed and placed in a 125 °C pre-warmed heater block (Hach-Lange, LT200) for a 60-minute extraction time wherein contents were vortex mixed briefly at 0, 5, 15, 30, 45, and 60 min. The 125 °C extraction temperature was selected based on mini-extraction experiments performed in 120 μL sealed crucibles and assessed with DSC measurements (described below).

After the 60-minute extraction time, the heater block was cooled to just below the solvent boiling point, and the biomass was allowed to settle in the tube. Aliquots of the solution supernatant were dispensed to crucibles for TGA or DSC analyses (see analytical methods), transferred for dilution into heated DMC for solution rheology measurements, and/or transferred for weighing and drying. When transferring for weighing and drying, the solution was transferred to a clean tare weighed vessel (petri dish or aluminium pan) and film cast. The weight of the transferred solution was measured directly, and the solvent was then evaporated to constant measured final sample dry weight at 60 °C.

Added and removed masses of solids and solvent (± 0.1 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 the film casting, and the known total mass of extraction solvent used. Extraction yields were calculated relative to the amount of dried biomass added to the extraction tube and were based on the biomass and cast film PHA contents that were measured by TGA. The recovered total polymer extract chemical and thermal properties were further characterized by PyGCMS, DSC, and intrinsic viscosity by solution rheology (see analytical methods).

2.2. Analytical methods

Thermogravimetric analysis (TGA2, Mettler-Toledo), for PHA content with respect to total solids (TS) and volatile solids (VS) as gPHA/gTS and gPHA/gVS, and for thermal decomposition temperature (T_d), was performed as previously described [35]. Representative sub-samples of about 5 and 2 mg of dried biomass, or of recovered polymer, were used, respectively. Samples were disposed to the tared TGA crucibles either as a ground powder, a film fragment, or an aliquot of hot polymer-solvent solution. When the polymer sample was dispensed as a solution, the solvent was evaporated leaving a film in the crucible before the measurement. The TGA measurement method included estimation of sample residual moisture (or solvent) and PHA contents as well as the overall organic and inorganic fractions. Briefly, pre-weighed samples were inserted to the TGA at 80 °C with nitrogen purge gas at 50 mL/min. Temperature was increased (10 °C/min) to 105 °C and held for 15 min for moisture (or any minor residual solvent) evaporation. Temperature was then increased (10 °C/min) to 550 °C and held for 30 min. PHA mass could be estimated from the characteristic rapid mass loss peak assessed above the background rate of mass loss on the DTG versus time curve that occurred between 225 and 350 °C. At 550 °C the purge gas was changed to air at 50 mL/min. Sample ash content was estimated by the end point of weight loss at 550 °C in air atmosphere over the 30 min. Reference samples included a PHA rich biomass with known PHA content (45.1 ± 0.6 gPHA/gVS), and pure PHB (> 98% purity, Biomer, Germany). The instrument was calibrated based on Curie temperature with a nickel standard following the Mettler-Toledo instrument protocol. TGA measurement analysis was performed with Mettler-Toledo STARe evaluation software and inhouse analysis software written with MATLAB® (Mathworks Version 2020b).

Differential scanning calorimetry (DSC 3+, Mettler-Toledo) was also performed based on previously described methods [35] and with nominally 5 and 2 mg samples for dried biomass and recovered polymer samples, respectively. PHA content was estimated from TGA measurements on parallel subsamples. Enthalpies from integrated polymer melt and crystallization peaks are reported with respect to the weight of PHA in the sample. Weighed samples disposed to a tared vented crucible were inserted and held for 5 min at −70 °C with nitrogen purge gas at 50 mL/min. A first heat and quench cycle was applied to standardize the thermal history with heating and cooling at 10 °C/min to 185 °C and back to −70 °C. A subsequent limited set of melt and quench cycles were performed between −70 and 185 °C while always applying the same heating rate (10 °C/min) but with quench rates ranging from −1 to −100 °C/min.

In selected DSC measurements, samples were aged at room temperature after being melted and quenched. These samples were aged at room temperature for selected times of up to 1 week after which time the samples where again melted and quenched. In the aged sample, the first DSC heating melt cycle was used to assess the polymer crystallization development (ΔH_m) due to the time allowed for secondary crystallization at room temperature. Reference samples included pure PHB (>98% purity, Biomer, Germany), and an in-house PHBV standard (34 % wt. average HV content). The instrument was calibrated with pure zinc and indium standards according to the Mettler-Toledo instrument protocol.

DSC was also applied to assess PHA melting in solvent and PHA gel-solution behaviour. An aliquot of an already formed polymer-solvent solution with known polymer concentration, or defined weights of polymer or biomass and added solvent, were dispensed to a sealable tare weighed crucible (Mettler-Toledo nr. 26929). Crucibles were inserted to the DSC and held for 5 min at the start temperature (5 or 10 °C) with nitrogen purge gas at 50 mL/min. A set of heating and quench cycles were applied by heating at 10 °C/min to a maximum temperature (depending on the solvent), and quenching back to the start temperature at selected quench rates in the range from −1 to −100 °C/min. The applied maximum temperature in these studies ranged up to 140 °C. Enthalpies estimated from integrated melt and crystallization (gelation) peaks, during heating and cooling ramps, were referenced to the mass of PHA in the crucible. DSC measurement analysis was performed with Mettler-Toledo STARe evaluation software and inhouse analysis software written with MATLAB® (Mathworks Version 2020b).

In selected cases, DSC heat flow measurements were normalized for qualitative comparison of similar scans (heating or cooling ramps) on different samples. Normalization was as follows: A mean heat flow signal for the scan was estimated. Then the root of the sum of the squared differences (RSD) between mean and the heat flow signal was estimated. Finally, the scan values were then offset to be zero centred by the mean signal value, and the offset signal was normalized by the RSD.

Polymer weight average molecular mass (in kDa) was estimated by solution rheology for intrinsic viscosity [η] measurements (dL/g). Polymer solutions (between 6 and 12 mL) were generated by combining weights of PHA and DMC to result in defined concentrations in the order of 15 mgPHA/mL. Polymers could be dissolved in DMC as per the extraction methods in sealed tubes by heating with periodic vortex mixing up to 125 °C for 5 min, and cooling to 65 °C. Alternatively, supernatant polymer-DMC solutions from extractions were diluted with pre-heated DMC (80 °C) and then maintained at 65 °C. Viscosity of 5 mL aliquots of the heated solutions were measured at 60 °C with a concentric cylinder measurement system rotating at a 75 s^{-1} shear rate (Anton-Paar MCR102 with a CC17 standard measuring system). Viscosity was estimated from 10 second averaged torque values logged over 5 min for each sample. Relative viscosities were estimated with respect to replicate measurements of DMC without added PHA. The intrinsic

viscosity was calculated from the Solomon-Ciuta equation [36]:

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

where c is the PHA concentration (g/dL) in DMC, η_{sp} is the specific viscosity, and η_r is the relative viscosity. Validity of linearity assumptions for intrinsic viscosity values estimated with this equation were confirmed from viscosity measurements on solutions of 98 percent pure polyhydroxybutyrate (1325 kDa, Biomer) and PHBV, (245 kDa, 34 wt.% 3-hydroxyvalerate) up to concentrations of 40 mg/mL. Conversion of intrinsic viscosity to an interpreted weight average molecular mass was made with the Mark-Houwink equation [37]:

$$[\eta] = K \cdot M_w^{\alpha} \quad (2)$$

where K and α are empirical constants relating [η] (dL/g) to a weight average molecular mass M_w (kDa).

The Mark-Houwink constants were calibrated based on a parallel set of samples dissolved in chloroform and with molecular weight determined by gel permeation chromatography (GPC) with GPC calibration to polystyrene standards following previously reported methods [35]. GPC measurements were undertaken with a 2xPL-Gel Mix-B LS column and OmniSEC Triple Detectors (refractive index, viscosity, and light scattering). All measurements were made at 35 °C with chloroform at a sample concentration of 3 mg/mL and at an elution rate of 1 mL/min. GPC column calibration was made with a set of 9 polystyrene standards distributed from M_n of 9 to 1200 kg/mol (Polymer Laboratories).

Polymer monomer composition was assessed by pyrolysis with gas chromatography and mass spectroscopy (PyGCMS) with adaptations from published methods [38–40] due to the available equipment. This evaluation gave the estimated weight percent of 3-hydroxyvalerate (3HV) in poly(3-hydroxybutyrate-co-3hydroxyvaleraye) (PHBV). A Gerstel (Mülheim an der Ruhr, Germany) pyrolysis unit was used with a Thermal Desorption Unit (TDU) and a liquid nitrogen Cooled Injection System (CIS). Grab samples of about 50 μg polymer particles, or PHA-rich biomass, were deposited into an open ended clean 25 mm long (Gerstel 018,131) quartz tube with a small retaining plug of quartz wool. Samples were loaded for pyrolysis by a Gerstel MPS 2XL autosampler. Pyrolysis was carried out under helium gas flow (60 mL/min) starting at 50 °C for 0.5 min followed by heating at 120 °C/min up to 710 °C for pyrolysis unit, and 350 °C for the TDU. The complete pyrolysis/TDU cycle lasted 8 min with maximum pyrolysis and TDU temperatures maintained. Pyrolysis products carried by the gas flow were trapped in the CIS at −100 °C on a quartz wool filled liner in solvent vent mode. Gas chromatography (GC, Agilent Technologies 6890 N, Santa Clara, CA, USA) was employed using a Phenomenex ZB-5MS column (30 m, 0.25 mm diameter, 1 μm film thickness). The CIS and GC temperature programs were run in parallel enabling transfer and focus of analytes onto the head of the column with a split flow of 20 giving 1 mL/min constant helium flow in the column. CIS temperature program was from −100 °C for 0.02 min, and then up to 280 °C at 600 °C/min. The GC oven temperature program was from 70 °C for 5 min, and then up to 290 °C at 10 °C/min. The detector after GC was an Agilent Technologies 5975 XL Mass Selective Detector (MSD). MS data were acquired in scan mode with mass-to-charge (*m/z*) ratios ranging from 15 to 550 in a total scan time of 0.649 s. Pyrolysis, TDU, and CIS were driven with Gerstel Maestro Controller (version 1.4.21.1) and MPS (version 1.4.15.1) drivers. GC and MSD were controlled by Evo3/Enhanced Masshunter GC/MS Acquisition (version B.07.06.2704). Acquired TIC (total ion chromatogram) data were processed and integrated using MassHunter Quantitative Analysis (Agilent Technologies, version B.07.00) while monitoring for the specific ion chromatogram (SIC) *m/z* 86 and 100. The SIC gave qualifying ions as markers for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate, respectively, since the pyrolysis process converts 3HB to 2-butenoic acid and 3HV to 2-pentenoic acid through a dehydration reaction [39]. NIST MS Search (version 2.0, 2008) was used to confirm the identity of 2-butenoic acid and 2-pentenoic acid. The integrated TIC peak ratio for 2-pentenoic acid with respect to the sum of 2-butenoic and 2-pentenoic acids was found to correlate directly to the 3HV wt% in PHBV using standards (Aldrich PHBV standards with 0, 5, 8 and 12 % wt. 3HV content) and for PHBV extracted using DMC. A correlation made between SIC and TIC integrated peaks from pure standards was then used to estimate the 3HV wt% for PHBV pyrolyzed within the more complex matrix of PHA-rich biomass samples. The SIC signal allowed to avoid interference from an overlapping signal that was present in the direct analysis of the PHBV composition in dried biomass samples.

Analyses of measurement data and trends including statistical analyses were performed with Microsoft Excel (for Mac Version 16) and Graphpad Prism (Version 9)

2.3. Modelling of random scission molecular weight loss

Thermal treatment of PHA is expected to result in random chain cleavage wherein molecular weight loss proceeds without initially generating volatile fragments [41]. In absence of measurable polymer volatilization, the rate of random scission is expected to follow a simple function of the number average degree of polymerization (P_n) [30]:

$$\frac{1}{P_{nt}} - \frac{1}{P_{n0}} = k_d \cdot t \quad (3)$$

$$\frac{P_{nt}}{P_{n0}} - 1 = (P_{n0} \cdot k_d) \cdot t \quad (4)$$

$$\frac{M_{n0}}{M_{nt}} - 1 = r_{sn} \cdot t = n_{sn} \quad (5)$$

where P_n is the number average degree of polymerization as a function of time (t), P_{n0} is the initial number average degree of polymerization ($t = 0$), and k_d is a random scission rate constant. M_{n0} and M_n are similarly the initial and time dependant number average molecular mass. r_{sn} is a scaled scission rate constant from the equation rearrangement and n_{sn} is defined as a scission number based on the number average molecular mass. A scission number n_{sn} increasing from 0 to 1 indicates successive chain cleavage to a number average molecular mass of half the initial value. A scission rate (r_{sw}) and number (n_{sw}) with respect to weight average molecular mass (M_w) can be similarly defined as a function of weight average molecular mass:

$$\frac{M_{w0}}{M_{wt}} - 1 = r_{sw} \cdot t = n_{sw} \quad (6)$$

$$\frac{M_w}{M_n} = .. \quad (7)$$

If Đ, the polydispersity, is constant, then n_{sn} and n_{sw} should follow the same trend as a function of time. The scission rate and number with respect to M_w can be estimated directly from intrinsic viscosity given the constant α from the Mark-Houwink Eq. (2):

$$\frac{M_{w0}}{M_{wt}} - 1 = \left(\frac{[\eta]_0}{[\eta]_t}\right)^{1/\alpha} - 1 = r_{sw} \cdot t = n_{sw} \quad (8)$$

The coupling between n_{sn} and n_{sw} was evaluated from GPC measurements on polymer samples extracted from biomass after heat treatment at 180 °C. Interpretations of the observations for molecular weight loss were made from results of numerical simulations of random scission.

2.4. Monte Carlo simulation of PHA molecular weight loss by random scission

Random scission was simulated for a starting molecular weight

distribution (MWD) of a homopolymer of polyhydroxybutyrate (M_1 = 86 Da). The initial MWD was defined by selecting M_w and Đ for a lognormal molecular weight distribution [42]. The total starting number of molecules in the MWD was generated by forcing the peak MW to be represented by 1000 molecules. For example, a starting M_w of 500 kDa resulted in a distribution of about 3.49×10^{10} molecules containing 3.82×10^{14} bonds. This distribution would be represented by polymer chains in the MWD with degree of polymerization in the range between 100 and 10,000. Constant cleavage rate due to thermal treatment was simulated based on a Monte Carlo approach [43]. Chain cleavage was applied in steps of sequential 1000 random scission events. For every iteration of 1000 events, the parameters describing the trend of MWD change (M_w, M_n, Đ, n_{sw}, n_{sn}) were logged. Each iteration step therefore defined an interval on an arbitrary time scale for an average scission rate with 1000 scission events per interval.

Each scission event required to select a bond to be broken on one parent molecule within the total distribution of molecules. Breaking the selected bond removed the parent molecule and resulted in two daughter molecules with respective chain lengths depending on the parent molecule chain length and the location of scission [43]. Two distinct models of random scission were evaluated – A. "random bond selection and scission", and B. "random molecule selection with centre weighted scission".

A Random bond selection was performed by randomly selecting a bond within the population of bonds defined by all the molecules present before each event. The parent molecule owning the randomly selected bond was identified along with the selected bond location on the polymer chain. The selected bond was removed to generate two daughter molecules.

B Random molecule selection with centre weighted scission similarly started by randomly selecting a bond, identifying the parent molecule owning that bond, and identifying its chain length. Therefore, the inherent probability of a scission event occurring on a given molecule was the same for both random scission models (A and B). In both cases, molecules with longer chain lengths, and present in higher numbers, inherently would have a higher probability of a scission event because they represented a larger fraction of the total bond population. Once the randomly selected molecule was identified, a bond for scission was selected based on a random number generated from a Gaussian probability distribution function. An output random number of zero would result in the most central bond on the selected molecule to be broken. Thus, the Gaussian distribution probability determined how far away from the centre of the molecule that scission would occur for that scission event. In this way, bonds closer to the centre of the molecule were more likely to be broken for the selected molecule. The Gaussian distribution was scaled to the selected molecule length to represent a constant number of standard deviations in any given simulation run. Stringency for centre weighting for scission could in this way be tightened or relaxed for each simulation run by using a narrower or a broader Gaussian distribution scaling with respect to molecule length.

Random scission simulations were performed with the model algorithms written in MATLAB® (Mathworks Version 2020b) using random number generation seeded by the date and time at the start of the simulation execution and with initialization by the Mersenne Twister generator.

3. Results and discussion

Dimethyl carbonate (DMC) was used as the extraction solvent for the purposes of this investigation. Already, more than 20 years ago, DMC was listed as a possible "PHA-poor" solvent that could be used for PHA extraction from biomass at elevated temperatures [44]. DMC has had renewed focus in proposed green solvent extraction with industrial scale recovery of PHAs from biomass [45,46]. A robust DMC extraction and polymer characterisation workflow was required to study effects of thermal pre-treatment as a pre-extraction molecular weight control strategy. Therefore, workflow methods were first developed and validated.

The presentation of results and discussion follows this progression of development from first building an approach in the workflow, and then to the practical evaluations to study the ideas of molecular weight control. Assessments for an effect of molecular weight change on the polymer thermal properties were then considered in epilogue. Firstly, a minimum applicable extraction temperature was determined by measuring polymer melt temperatures in solvent by DSC. Gelation tendency of PHBV in DMC over a wide range of 3HV contents at 50 gPHA/L was then characterized also by DSC. From this characterization, a sufficient conservative temperature was selected to maintain stable polymer-solvent solutions that were required for intrinsic viscosity determinations in DMC. M_W interpreted from intrinsic viscosity measurements in DMC were then calibrated from a set of parallel measurements made by GPC in chloroform. Numerical simulation of random scission due to thermal treatment then enabled to interpret practical observations of molecular weight distribution change during heat treatment experiments. Replicate experiments were then made to evaluate a potential for MW quality control by biomass pre-extraction thermal treatment. These experiments covered a range of distinct biomass batches containing PHBV with different monomer compositions. Finally, from the complete dataset, assessments were made for evidence of any effect of the thermal pre-treatment on extraction yields, and the polymer thermal properties.

3.1. Extraction temperature selection and validation for DMC

DMC extraction of PHA from biomass has been recently performed at the solvent boiling point of 90 °C [47,48]. However, for the present work, 90 °C was found to be insufficient for generic routine extractions over a wide range of PHBV types and with thermal dried MMC PHA-rich biomass. The dissolution temperature and kinetics of PHBV extraction in DMC, as for other PHA-poor solvents, is a function of crystallinity. Specifically, higher extraction temperature is required for PHBVs with higher crystallinity. The crystallinity of PHBV is influenced by 3-hydroxyvalerate (3HV) average content (the higher the 3HV content up to a eutectic point, the lower is the crystallinity) and its distribution [35]. Dissolution temperatures of PHBVs in solvents were assessed by DSC in the sealed unvented crucibles. Mixtures of weighed amounts of solvent with weighed amounts of purified PHBV, or PHA-rich biomass, were studied. Typical results are shown in Fig. 1.

PHB is a worst-case scenario for extraction due higher crystallinity. Neat commercial PHB powder (0.98 gPHA/gTS, 1350 kDa) melted between 121 and 179 °C, with a median melt temperature (T_{50}) of 172 °C, and ΔH_m of 100 J/gPHA in the first melting ramp. A median melt temperature is reported because, in general, PHBV co-polymer blends with increasing average 3HV content can exhibit broad melting peaks [18]. Melting of commercial PHB in DMC was assessed for concentrations of 50 and 100 mgPHA/gDMC. The observed melting peak in both samples was similarly between 90 and 120 °C with a T_{50} of 105 °C and a melt enthalpy (ΔH_m) of 60 J/gPHA. For comparison, the same PHB melted in 2-butanal (BOH) at 85 mgPHA/gBOH, but at a higher temperature range from 112 to 131 °C, a T_{50} of 122 °C, and a ΔH_m of 64 J/gPHA. PHA-poor solvents enable the polymer to melt into solution at lower temperatures and with lower enthalpy than the neat polymers. The solvent diffuses into the polymer, swells the microstructure, and weakens crystallinity. From DSC evaluations, minimum temperatures of 120 and 131 °C may be interpreted to be necessary to reliably extract pure PHB in DMC and BOH, respectively.

The extraction melt temperatures were also readily measurable in PHA-rich biomass by DSC (Fig. 1). Biomass (a) with 0.35 gPHBV/gTS (4 wt.% 3HV) exhibited a T_{50} and ΔH_m of 109 °C and 42 J/gPHA in DMC

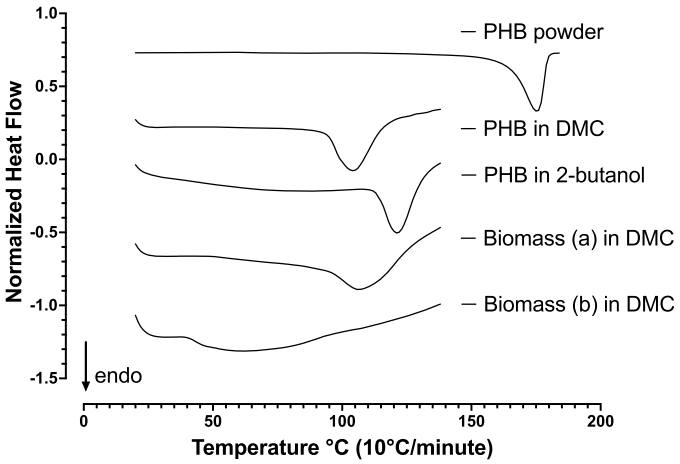

Fig. 1. Examples of normalized heat flow showing DSC melting peaks in a first heating ramp for PHB powder and PHBV co-polymer blends in biomass with or without presence of solvent (dimethyl carbonate – DMC, or 2-Butanol). PHB powder: 0.98 gPHB/gDS and 1350 kDa. Biomass (a): 0.35 gPHBV/gTS and 4 wt.% 3HV. Biomass (b): 0.42 gPHBV/gTS and 36 wt.% 3HV.

(50 mgPHA/gDMC). Biomass (b) with 0.42 gPHBV/gTS and 36 wt.% 3HV gave a T_{50} and ΔH_m of 65 °C and 18 J/gPHA in DMC (168 mgPHA/gDMC). Lower melt temperatures and enthalpies indicated the ability for extraction at lower temperatures given lower average crystallinity with increased average 3HV co-monomer content. This outcome can give a reason why 90 °C can be, but may not have always been, a sufficient temperature to efficiently extract all PHBV types, or blend fractions, for co-polymer blends from biomass in the selected mentioned cases reported above in the cited research literature.

Taking PHB as a worst-case scenario, 125 °C (1 h) was selected as a conservative minimum standard DMC extraction temperature based on the DSC evaluations. DMC extractions at 125 °C required to safely manage solvent overpressure in the closed extraction tubes. From Antoine coefficients [49] the DMC overpressure was estimated to be 2.8 bar at 125 °C.

Generic suitability of 125 °C (1-h) was validated with 13 biomass samples comprising a range of biomass PHBV contents (from 0.36 to 0.55 gPHA/gTS), a range of 3HV co-polymer compositions (2 to 41 average wt.% 3HV), and consistent high thermal decomposition temperature (T_d = 285 ± 2 °C, 10 °C/min). A T_d in this range has been previously found to be important towards mitigating excessive molecular weight loss during extraction [34]. With consistently high T_d, the extent of molecular weight loss given the same extraction conditions, has been found to be similar, and independent of PHBV 3HV content and initial MW [18]. The applied extraction loading was 50 ± 5 mgPHA/gDMC (n = 13). Mass balances gave an average extraction yield of 106 ± 5 percent with a total extract PHA purity of 95 ± 2 percent. The consistent extraction outcomes validated a benchmark method that was insensitive to variability of biomass PHA content, polymer composition, and molecular weight range for these samples.

Extraction yields were slightly but nevertheless positively affected by higher biomass PHA content or higher average 3HV content. The average mass balance overestimation of extraction yield (106 percent) could suggest a minor systematic underestimation of about 0.02 gPHA/gTS for the biomass PHA content determination by TGA. This underestimation would represent a minor mass of the polymer that is not easy to distinguish from the background non-polymer biomass (NPB) volatilization during TGA. Replicate TGA measurements for one of the biomass samples gave a precise average content of 0.350 ± 0.006 gPHA/gTS (n = 10). These replicate measurements were also independent of a systematically applied sample size variability from 2 to 20 mg, while 5 to 10 mg dried solids were typically used for TGA routinely. The TGA results of estimated PHA content for the same 13 biomass samples were not significantly different (P = 0.3225) from independently performed round-robin measurements made by sample digestion and gas chromatographic methods [50] with an outcome of mean difference for same biomass samples (TGA-GC values) of 0.006±0.021 gPHA/gTS. An error of 0.02 gPHA/gTS was therefore within an expected accuracy of the measurements of biomass PHA content in general and this fits with previous experience [35].

On average, the measured extracted PHA concentration was directly proportional to the applied extraction loading. About 3 ± 1% (n = 13) of the NPB suspended solids were solubilized and co-extracted in DMC at 125 °C (1-h). A higher biomass PHA content will therefore result in a total extract of higher polymer purity in general. For these biomass samples, higher average 3HV content tended to result in slightly less co-extracted NPB.

These results offer extension to published findings for PHA extraction with DMC at 90 °C [47,48,51]. A higher DMC extraction temperature can enable complete generic extraction of PHBVs from acid stabilized [34], convection oven dried, mixed culture (municipal activated sludge) biomass for routine evaluations of total extractable polymer.

3.2. *Avoiding polymer gelation for solution rheology measurements with DMC*

It was a goal in the workflow to be able to measure the extracted polymer molecular weight directly by solution viscometry [52] in DMC. Gelation needed to be avoided [53–55]. The gels are structures of the solvated polymer that form upon cooling such polymer solutions. They are maintained as a three-dimensional network of crystallized zones, for which characteristic cooling formation and heating melt temperatures and enthalpies can be evaluated by DSC in sealed crucibles. As part of the extraction validation experiments for 13 biomass samples (Section 3.1), an aliquot of still hot but cooled (<90 °C) dissolved polymer solution, after extraction and separation from the non-dissolved biomass solids, was transferred directly to DSC crucibles. Crucibles were then directly sealed, cooled to room temperature, and left to stand overnight before analysis by DSC. The first heating ramp gave a median gel melting temperature of 66 ± 9 °C (n = 13) and the trend in enthalpy of the melting peak indicated a loss in gelation tendency in DMC beyond a 3HV average content of about 20 wt percent (Fig. 2a). Nevertheless, two samples with high 3HV PHBV, resulted in observed gel melt enthalpy levels outside of the interpreted dominant trend. This trend was estimated as a second order polynomial decaying from a peak value for PHB. It has been previously shown that these PHBVs are a miscible blend of distinct fractions that are separable due to changes in solubility of individual molecules as a function of average 3HV content or molecular weight [18]. Such selective fractionation precipitation occurs when adding hexane to co-polymer blends dissolved in chloroform at room temperature [56]. Solubility of PHAs changes in DMC due to change in temperature. Therefore, it was interpreted that, given sufficient time, selected co-polymer fractions within the polymer blend may still form gels or precipitates notwithstanding the total blend average 3HV content. Measurable exothermic spontaneous gelation did not occur for PHBVs with over 10 wt percent average 3HV for these PHBV co-polymer blends when cooling the solutions at −1 °C/min directly after melting the gels by heating up to 125 °C (Fig. 2b).

Both gelation onset temperature and enthalpy increased with decreasing average 3HV content. Therefore, gelation of neat PHB in DMC was evaluated as a worst-case scenario for interests of avoiding gelation, in general, during polymer-DMC solution viscosity measurements. Onset gelation temperatures were determined as a function of quenching rate for PHB solutions at nominally 50 mgPHA/gDMC and with commercial PHB (0.98 gPHA/gTS, 1350 kDa). For comparison, a previously produced batch of pilot scale extracted and washed [18] PHBV with 34 wt.% 3HV (0.98 gPHA/gTS) was also evaluated for its gelation tendency. A series of quenching rates were applied for the polymer solutions (see Fig. 3) cooled from a maximum temperature of

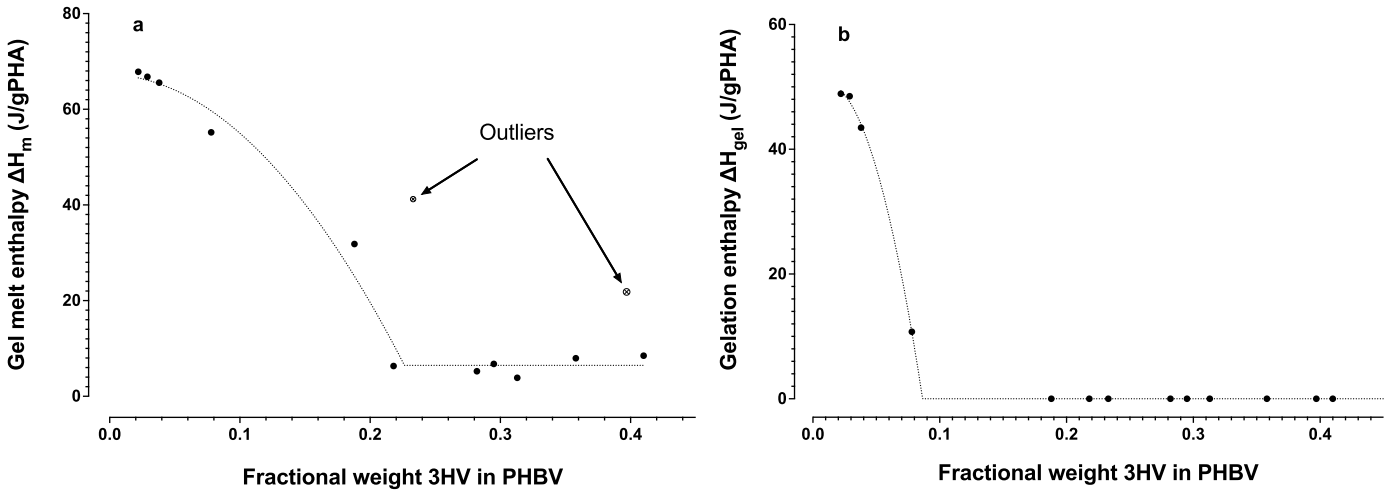

Fig. 2. Behaviour of PHBV characterized with average weight percent 3HV and extracted into DMC at nominally 50 mg/gDMC: (a) expressed gel melt enthalpies upon re-heating cooled solutions at 10 °C/min upon standing at room temperature, and (b) expressed gelation enthalpies upon cooling freshly melted solutions at −1 °C/min.

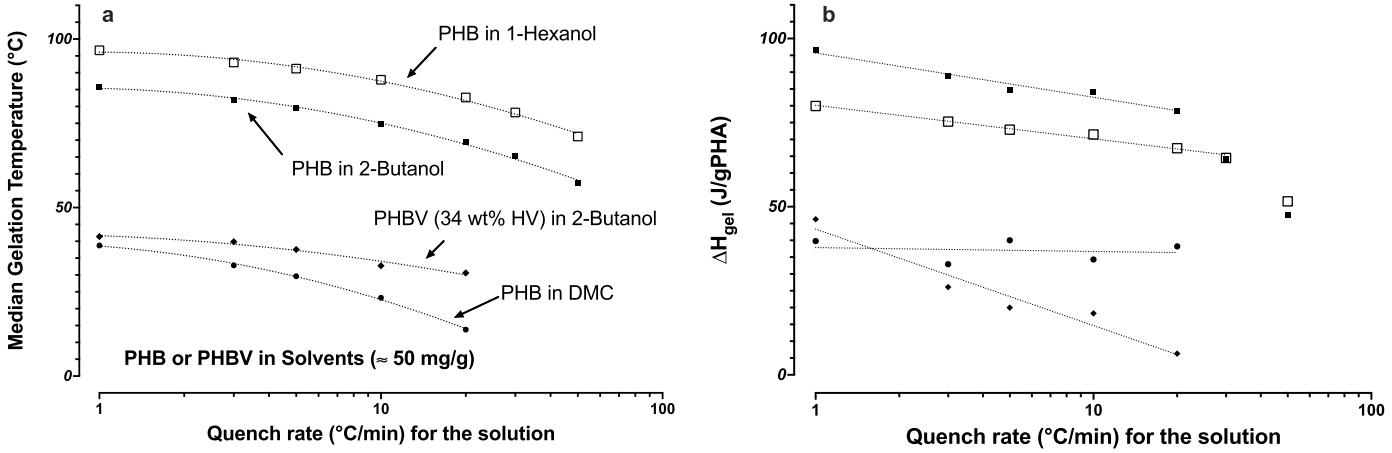

Fig. 3. Median gelation temperatures for PHB and PHBV (34 wt.% 3HV) in DMC, 2-butanol, and 1-hexanol as a function of quench rate from polymer solutions at elevated temperatures (a). Corresponding exothermic gelation enthalpies (b).

100 °C (DMC), 120 °C (2-butanol), or 140 °C (1-hexanol). This maximum temperature was determined to be above the gel melting temperature in all cases. The combination of co-polymer type and solvent influences the driving force and temperature for gelation (Fig. 3a). The extrapolated isothermal gelation temperature of the PHBV in 2-butanol was found to be like outcomes of PHB in DMC. However, it could also be seen that this PHB gelled more slowly in DMC versus the PHBV in BOH as indicated by lower observed gelation temperatures with faster quench rates for similar concentrations. An average enthalpy of gelation of the PHB was 37 ± 3 J/gPHA (n = 5) with no significant correlation to quenching rate (Fig. 4). No gelation enthalpy was measurable for the 34 wt.% 3HV PHBV in DMC.

In contrast, gelation enthalpy was quench rate sensitive for the PHB and PHBV in 2-butanol indicating an influence of quenching rate on gel formation. The average gelation enthalpy for PHB in 2-butanol was estimated to be 78 ± 7 J/gPHA. Thus, 2-butanol is a "poorer" PHA-poor solvent. Reduced solubility of PHBV in BOH (versus DMC) with temperature, supports that faster cooling of PHA solutions in BOH will more quickly restrict polymer mobility during gelation and, in so doing, influence the resulting quenched gel structure [53–55].

An isothermal onset gel crystallization temperature for the commercial PHB in DMC at 50 mgPHA/gDMC was estimated to be 47 ± 1 °C (Fig. 4). This value was obtained from least squares regression analysis

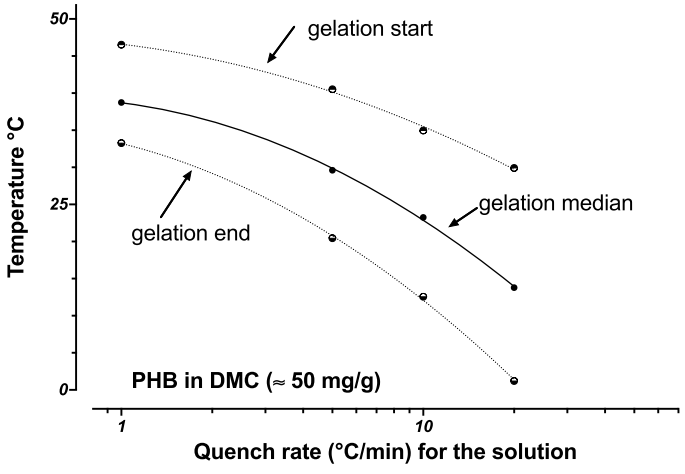

Fig. 4. Start median and end temperatures for an exothermic gelation peak from DSC with PHB in DMC as a function of quench rate from polymer solutions at elevated temperature.

to the second order trends as shown in Fig. 4, for the gelation start temperature versus the logarithm of quench rate. To avoid gelation during rheology measurements, 60 °C was selected as a conservative and workable temperature for routine assessments of polymer-solvent solution viscosities at concentrations between 10 and 25 mgPHA/mL. Tendency for the formation of a gel as PHA solutions cool in PHA-poor solvents decreases with decreasing concentration [22]. Solutions were generated by first melting the polymer into solution at 125 °C for a few minutes, then cooling directly down to between 60 and 70 °C pending viscosity measurements.

3.3. Mark-Houwink constants for PHBV intrinsic viscosity in DMC at 60 °C

Mark-Houwink constants (Eq. (2)) were estimated from DMC polymer-solution intrinsic viscosity measurements. A systematic range of polymer MWs was generated by applying the proposed pre-extraction thermal treatment in air. Controlled molecular weight loss was achieved by an isothermal heat treatment at 180 °C on 12 subsamples from the same batch of a dried PHA-rich biomass for up to 120 min before subsequent direct DMC extraction. This biomass contained PHBV at 0.43 gPHA/gTS and with 36 wt.% 3HV. Heat treatment resulted in up to a 7 percent biomass weight loss over the incubation time. This loss corresponded to a PHA content increase of 2 percent. Mass balance assessment indicated that heat treatment mass losses were due only to NPB volatilization. The heat treatment was not found to affect the polymer extractability in DMC at 125 °C (M_w range 50 to 500 kDa – see below). A consistent yield and total extract purity was again independently obtained, 104 ± 5% and 96.5 ± 0.4% (n = 12), respectively, as was found for the 13 distinct batches reported above.

The decreasing trend of extracted polymer intrinsic viscosity in these 12 samples as a function pre-extraction isothermal incubation time is shown in Fig. 5a. Replicate intrinsic viscosity measurements gave reproducible results (see 0, 30 and 120 min of incubation in Fig. 5a). The trend reflected kinetics of expected time-temperature dependant polymer average molecular weight loss by random scission [30–33,41,57]. Corresponding seven-point calibration (r^2 = 0.9994) between intrinsic viscosity [η] and weight average molecular mass (M_w) measurements by GPC were fitted with the Mark-Houwink (MH) equation (Fig. 5b). The α and LogK values were 0.74 ± 0.01 and −2.016 ± 0.025, respectively, for Eq. (2).

The estimated MH exponent, α = 0.74, for DMC at 60 °C was like α values of 0.82 and 0.84 for a "random coil", obtained for PHB in chloroform at 30 °C and TFE at 25 °C, respectively [37,58]. Accounting for MW expressed in kDa, K values are different between the current study and the literature values, as may be expected, due change of polymer, solvent, and temperature. Intrinsic viscosity measurements with chloroform have been used in the MMC literature to evaluate MW of extracted PHBVs in general [27,59,60]. However, an influence of MH parameters from changes in PHBV composition has not been systematically studied. In general, there can be uncertainty when comparing MW values derived and interpreted by different methods and equipment [61]. One recent study suggests that large differences in 3HV content will not have a strong influence on the MH constants [62]. In the present work it was found that interpreted M_w estimated from intrinsic viscosity for the 13 benchmark samples (Section 3.1), were not significantly different (ratio pairwise t-test, 95% confidence interval) with a geometric mean of ratios of 1.104 and with standard deviation of the log (ratios) of 0.1421 (n = 13). Differences in MH constants for the different PHBVs could have contributed to observed variability in this comparison, and the significance of PHBV composition effects on the MH constants requires further investigation. In the present work it was most of interest to follow trends of scission number. These trends were estimated with intrinsic viscosity using Eq. (8), an α of 0.74, and the assumption that this α obtained for DMC at 60 °C could be representative for the PHBV co-polymer blends evaluated in this work.

Polydispersity index values estimated from GPC (Eq. (7)) gave an initial PDI of 2.11 and evidence of a statistically significant (p<0.0001) but still small linear decay rate in PDI over the incubation time (−0.0024 ± 0.0005 min^{-1}) as shown in Fig. 5a. PHA accumulation in biomass granules is by chain elongation for which a maximum polydispersity index of about 2 is theoretically expected [63].

3.4. Number average versus weight average scission numbers and rates

The molecular weight distributions for extracted PHBV, with or without heat treatment, fitted well to a lognormal peak (Fig. 6a). Thermal treatment of PHA is expected to promote random chain cleavage wherein molecular weight loss proceeds without initially generating volatile fragments leading to sample total weight loss [41]. PHBV co-polyesters are generally found to be unstable over 170 °C with temperature dependence for scission kinetics independent of co-polyester composition [64]. Given that the biomass isothermal treatment was not found to result in any significant measurable polymer volatilization, the rate of random scission was expected to follow a simple linear trend based on the number average degree of polymerization [30]. Scission number (n_{sn} or n_{sw}) increased at a constant rate after a short induction period of 8.6 min (Fig. 6b). Since scission rate is

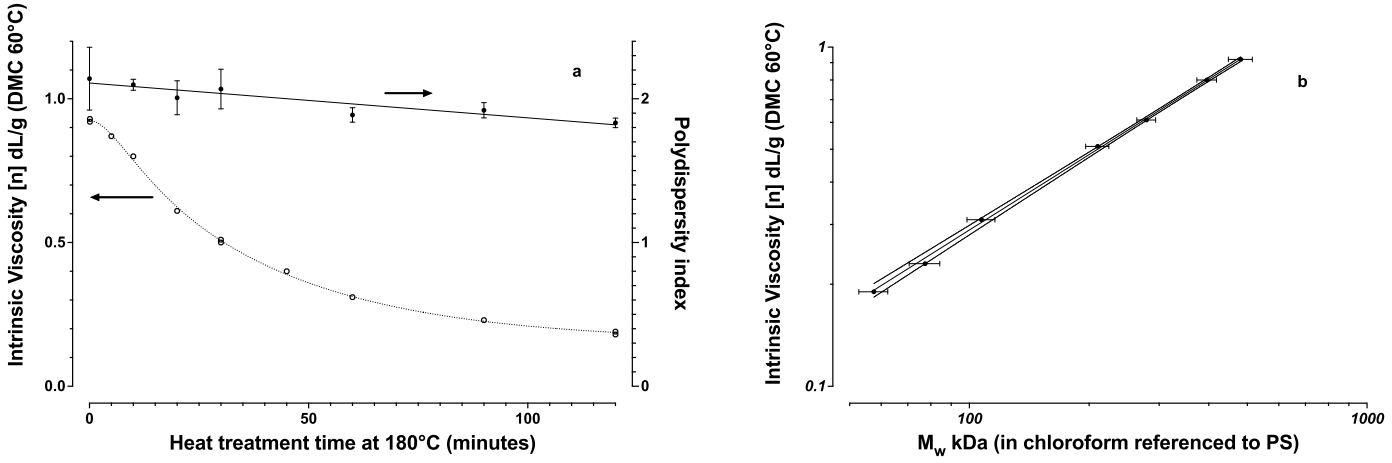

Fig. 5. Trend of extracted PHBV (36 wt.% 3HV) intrinsic viscosity (open circles) in DMC following isothermal heat treatment of biomass at 180 °C just prior to extraction (a). Intrinsic viscosity levels correlated, with the Mark-Houwink equation, to replicate measurements of weight average molecular weights (n = 5) measured for the same samples in chloroform (b). PDI for the polymer decreased slightly (closed circles) but significantly over time based on the GPC results (a).

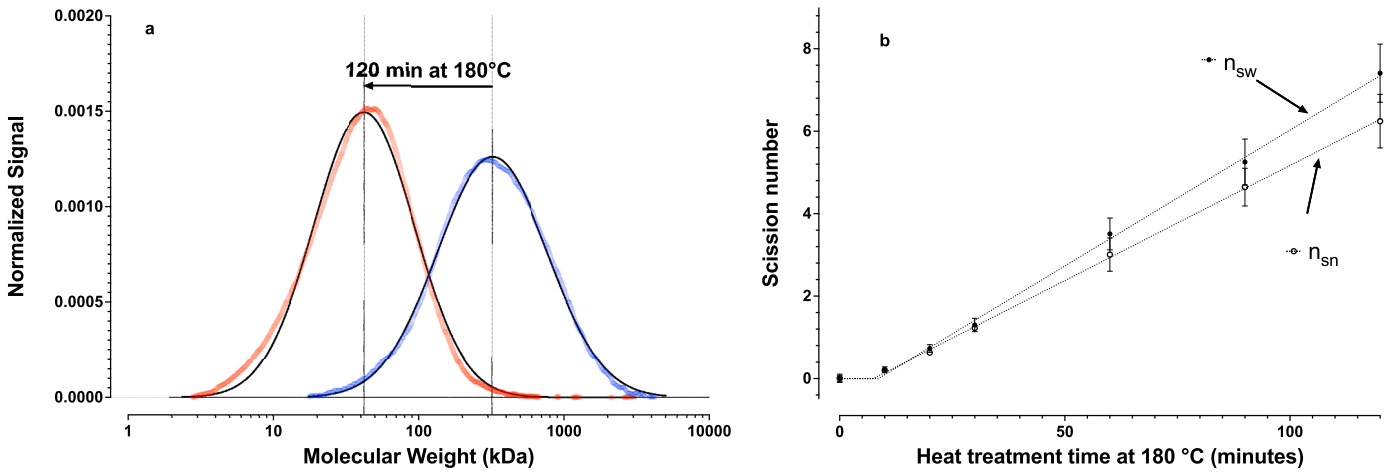

Fig. 6. Sustained lognormal molecular weight distribution for DMC extracted PHBV before and after 120 min 180 °C heat treatment of biomass before extraction (a). GPC data are shown with best fitted distributions (solid lines) by non-linear regression analysis. Development of weight and number average scission numbers (n_{sw} and n_{sw}) based on GPC measurements as a function of heat treatment time before DMC extraction (b).

expected to follow an Arrhenius temperature dependence [32], slower initial cleavage rates may have been due to a sample mass heating lag time. A constant scission rate was attained as expected. However different random scission rates with respect to number and weight average molecular mass resulted (r_{sn} and r_{sw}). These rates differed due the significant rate of change of PDI for this sample (Fig. 5a).

A polymer weight average molecular weight half-life was defined as time required for the polymer to reach a scission number n_{sw} equal to 1. The molecular weight half-life for the polymer in this biomass sample was therefore only 14.4 min at 180 °C in air, neglecting the interpreted initial heat up lag time of 8.6 min. The results suggested that molecular weight of the polymer in PHA-rich activated sludge can be predictably degraded to a targeted lower value prior to extraction. Lower temperature heat treatment would require longer incubation times to reach the same reduced M_w. For industrial scale operations, lower heat treatment temperature may be preferred. Lower temperatures for slower rates of

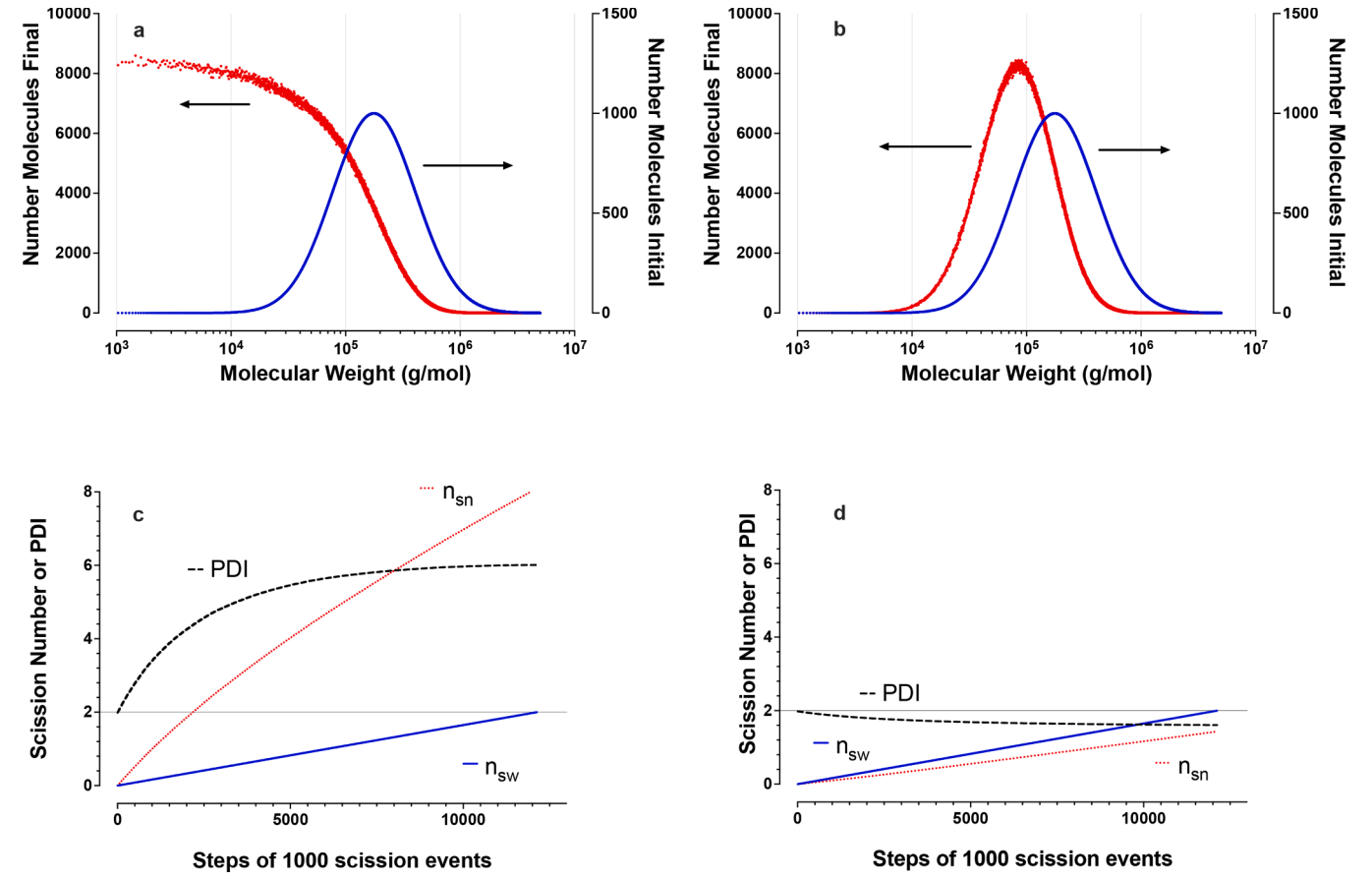

Fig. 7. Final and initial molecular weight distributions for Monte Carlo simulation of random bond selection and scission of a lognormal PHB starting at 500 kDa and PDI equal to 2 (a). The same as for (a) but for random molecule selection with centre weighted scission Z-score ± (b). Trend in scission numbers (n_{sw} and n_{sn}) as well as polydispersity indices for simulations (a) and (b) are shown in (c) and (d), respectively.

molecular weight loss may avoid grossly undershooting, or overshooting, the targeted endpoint MW if precision is relatively poor at large scale for the heat treatment process time control.

3.5. Cleavage simulation and robustness of reporting scission rates with respect to n_{sw}

Random scission rates have been typically described quantitatively in the research literature with respect to the number average degree of polymerization Eq. (5)). However, for the current and for ongoing investigation, it was desirable to define the scission rate based on the weight average scission number (n_{sw}) using routine intrinsic viscosity measurements. A challenge was that an interpretation of n_{sw} was not known as the founding developments are based on number average molecular weight changes (Eqs. (3) and (4).

Numerical simulations of random polymer scission were undertaken to see if it was possible to reproduce the observed trends found for both n_{sn} and n_{sw} in Fig. 6. Simulations of molecular weight loss were applied to a lognormal polymer distribution with initial M_w and PDI of 500 kDa and 2, respectively. The MWD was generated with 1000 molecules at the peak and for the homopolymer PHB with M_1 of 86 Da (Figs. 7a or 7b). Simulations were made with the different "A and B" definitions of "randomness" of scission events at a constant applied scission rate. These two approaches of randomness are presented in the Materials and Methods section. The numerical simulations of molecular weight change were all made in repeated steps of 1000 scission events and the simulations were terminated when n_{sw} reached 2.

The case of (A) "random bond selection and scission" did not represent the practical experimental observations (Fig. 7a and 7c). Low molecular weight monomers, dimers, trimers, and oligomers were rapidly generated. PDI increased asymptotically from 2 to 6 (Fig. 7c). Notably, r_{sw} was constant, but r_{sn} was asymptotic towards a linear trend. In contrast, the practical experiment observations (Fig. 6) were reproduced for the case of (B) "random molecule selection with centre weighted scission" (Fig. 7b and 7d). Increased stringency of central scission location probability for randomly selected molecules was made by increasing the Z-score scale to define the Gaussian probability distribution of scission location over the molecule length. For example, at a Z-score of ± 3, for the normalized molecule length, there would be a 68.2% probability that cleavage should occur for bonds within the central third of each molecule.

At a Z-score scale of ± 6, the observed practical findings of constant r_{sw} and r_{sn} with a significant but minor decrease in PDI over time was simulated (Fig. 7d). The trends in Fig. 7d qualitatively mimicked the observations shown in Fig. 6. The simulations suggest but can not prove that there can exist a degree of inherent selectivity in the location for random scission events on polymer molecules during PHA-rich biomass thermal treatment.

It was most significant to observe that the weight average scission rate, $r_{sw,}$ was independent of the model assumptions. The ideas of stringency of central tendency for scission, or for the two models of random scission applied, did not influence the outcomes of r_{sw}. In contrast, r_{sn} was very sensitive to the conditions of randomness that were imposed in the simulations. Thus, simulation results with distinct models of randomness for chain cleavage events support that the weight average molecular mass scission number (n_{sw}) is a robust parameter to represent trends of scission rate as function of time. The simulation outcomes with respect to r_{sw} resulted in an outcome of polymer M_w half-life equal to about 6063 steps or $6.063{\cdot}10^6$ scission events. Therefore, the above measured r_{sw}, from the results reported in Fig. 6, can be related to the model simulation results. For the observed polymer half-life of 14.4 min, a scission event rate in the order of 421 042 scission events/min is estimated for that sample that was held at 180 °C. Numerical simulation data of r_{sw} can be used to estimate actual scission rates.

3.6. Evaluation of thermal pre-treatment for molecular weight quality control

Replicate heat treatment experiments were conducted with PHA-rich biomass subsamples from six of the 13 batches of PHBV-rich biomass with average 3HV contents of 4, 8, 19, 23, 31 and 36 wt percent. Heat treatment (180 °C and up to 60 min) in air was applied to the PHA-rich biomass subsamples in all cases, and the PHA was then extracted in DMC (125 °C, 1 h) as before. M_w values from intrinsic viscosity measurements of all the extracted polymers were estimated with the determined MH constants, and the trends of weight average scission number (n_{sw}) were evaluated with Eq. (8). In combination with the initial 13 non-heat-treated biomass samples, a matrix (Fig. 8) of samples spanning 3HV contents and weight average molecular mass was evaluated for polymer properties. The goal was to establish perspective on the generic potential for systematic control of molecular weight before PHA extraction, and to evaluate if molecular weight loss in combination with the polyester average 3HV content affected extractability or other polymer thermal properties.

For the complete data set ($n = 45$) including both heat-treated (up to 1 h) and non-heat-treated samples, extraction yield and purity were 100 ± 7 and 91±4 percent, respectively. On average 7 ± 3 percent of the NPB was co-extracted. The variability in extraction yields and purities did not correlate with the estimated extracted polymer M_w (80 to 1320 kDa), nor with the average PHBV 3HV contents (2 to 41 wt.%).

Scission rates were relatively constant in 3 out of the 6 cases (Fig. 9a, c and f). In the 3 cases where scission rate was not constant (Fig. 9b, d and e), both increasing and decreasing rates were observed. When the rate was not constant, it asymptotically approached a constant rate. The initial scission rates were not found to be correlated to the polymer in biomass thermal stability (T_d = 288±2 °C), biomass moisture content before extraction (0.8 ± 0.1 percent), the biomass PHA content, the polymer average 3HV content, nor the initial weight average molecular weight. Any underlying causal qualities of the biomass-polymer matrix for predication of cleavage kinetics could not be identified and requires further investigation. A change in the MH constant α (Eq. (8)), between the PHBV types tested and shown in Fig. 9 can also affect the interpreted n_{sw} scission number trend shape. An influence due to the MH converted MW cannot be ruled out without further investigation. If every type of PHBV requires a specific set of MH constants, then reliable conversion of

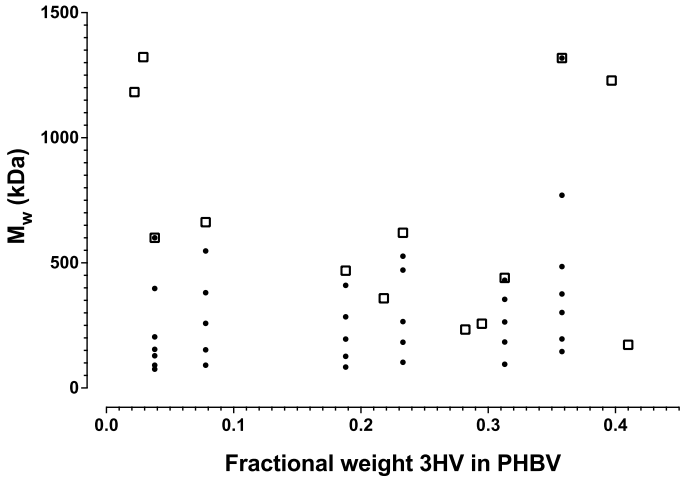

Fig. 8. Sample set of PHA-rich biomass from 13 individual accumulation batches (open squares), and from heat treatment of subsamples (180 °C in air before extraction) as performed for selected batches (closed circles), that were used and generated for the purposes of this investigation. Respective interpreted weight average molecular mass M_w was determined from intrinsic viscosity measurements with MH constants from Section 3.3, and 3HV content by pyrolysis GCMS measurements.

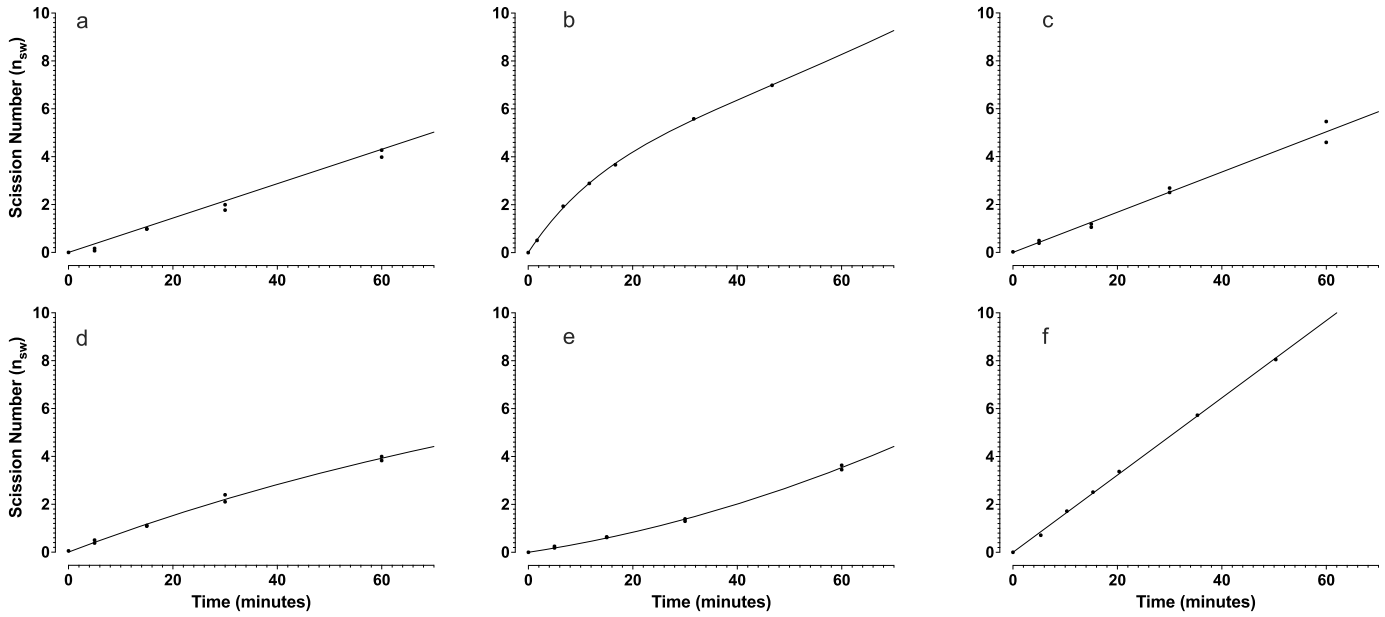

Fig. 9. Molecular weight loss trends reported as scission number (n_{sw}) for heat treated of PHA-rich biomass (180 °C in air up to 1 h) before extraction with DMC (125 °C, 1 h). Biomass samples covered a range of PHBV types, and interpreted initial weight average molecular mass as follows (% wt. 3HV, M_{w0} kDa): a(3, 526), b(4, 600), c(8, 548), d (19, 410), e(31, 430), f(36, 1318). Replicate reported measurements (in cases a, c, d, e) are separate samples with method steps of heat treatment, extraction, and subsequent polymer characterization.

intrinsic viscosity to M_w (calibrated to polystyrene) will become an onerous task. Alternatively, at least for the industrial process, MW quality control can also be based simply and reliably on reaching a targeted intrinsic viscosity level (Fig. 5a).

Even if absolute values and trends of scission rates were not found to be attributable to other measured properties, replicate samples given the same heat treatment for the same time resulted in reproducible outcomes (Fig. 9a, c, d, and e). Therefore, if the scission rate for a given PHA-rich biomass is characterized for a defined pre-treatment temperature history, then it is anticipated from this work that the interpreted M_w of the recovered polymer from that biomass batch can be reduced predictably to a targeted lowered value without affecting subsequent extraction outcomes.

3.7. Thermal pre-treatment effect on PHBV melt and crystallization properties

Melt temperatures and enthalpies of the extracted PHBVs, with and without thermal pre-treatment, were compared. Comparison required that all samples had the same thermal history and sufficient ageing for all samples to reach a stable level of crystallization [65]. Standardizing the polymer thermal history was through melting and quenching, and then ageing the sample for at least 3 days, but not more than one week. This ageing time frame was selected based on a practical evaluation with an observed trend of secondary crystallization for a PHBV with 34 wt.% 3HV content (Fig. 10a and b). The measured polymer melt enthalpy stabilized to a steady value after 48 h of incubation at room temperature following a standard applied melt and quench history before ageing.

Melting peaks were broader with increasing average 3HV compositions approaching the eutectic point. Due to such broad peaks, melting temperatures were characterized with onset, median, and end temperatures (T_{05}, T_{50}, and T_{95}) given by 5, 50 and 95 percent of the distribution, respectively (Fig. 11a). Broader melting peaks observed for the PHBVs with higher average 3HV content suggest for a broad range in distribution of 3HV containing molecules in the polymers that comprised these co-polymer blends. The applied thermal treatments

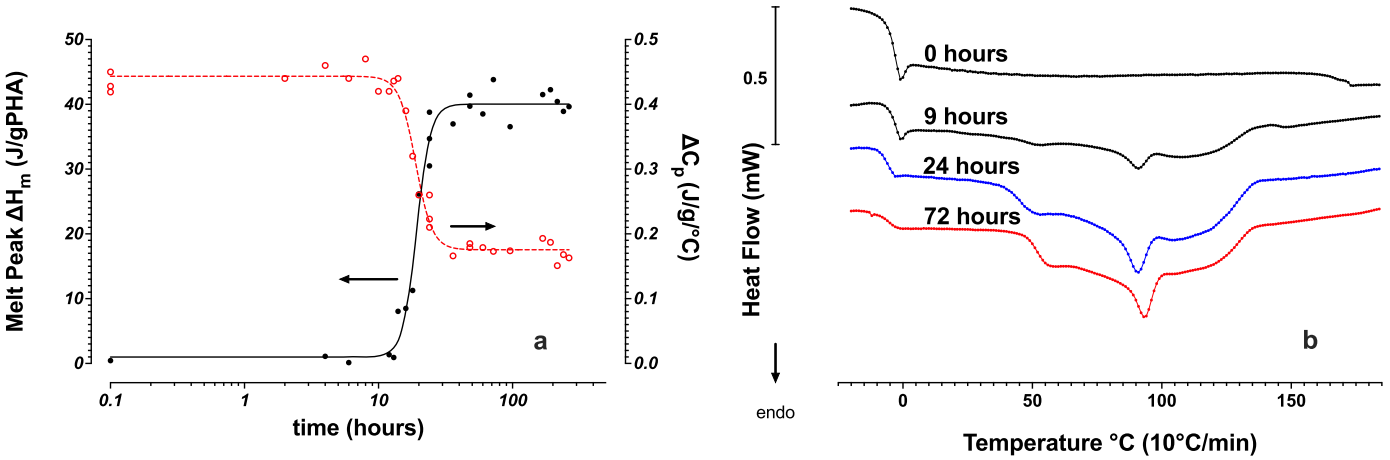

Fig. 10. Development due to secondary crystallization after a melt-quench cycle and the given time in hours of aging at room temperature (RT) for a PHBV with an average 3HV content of 34 wt.%. Glass transition ΔC_p and melt ΔH_m were evaluated after the RT ageing with a heating ramp starting from -30 °C at 10°C/min.

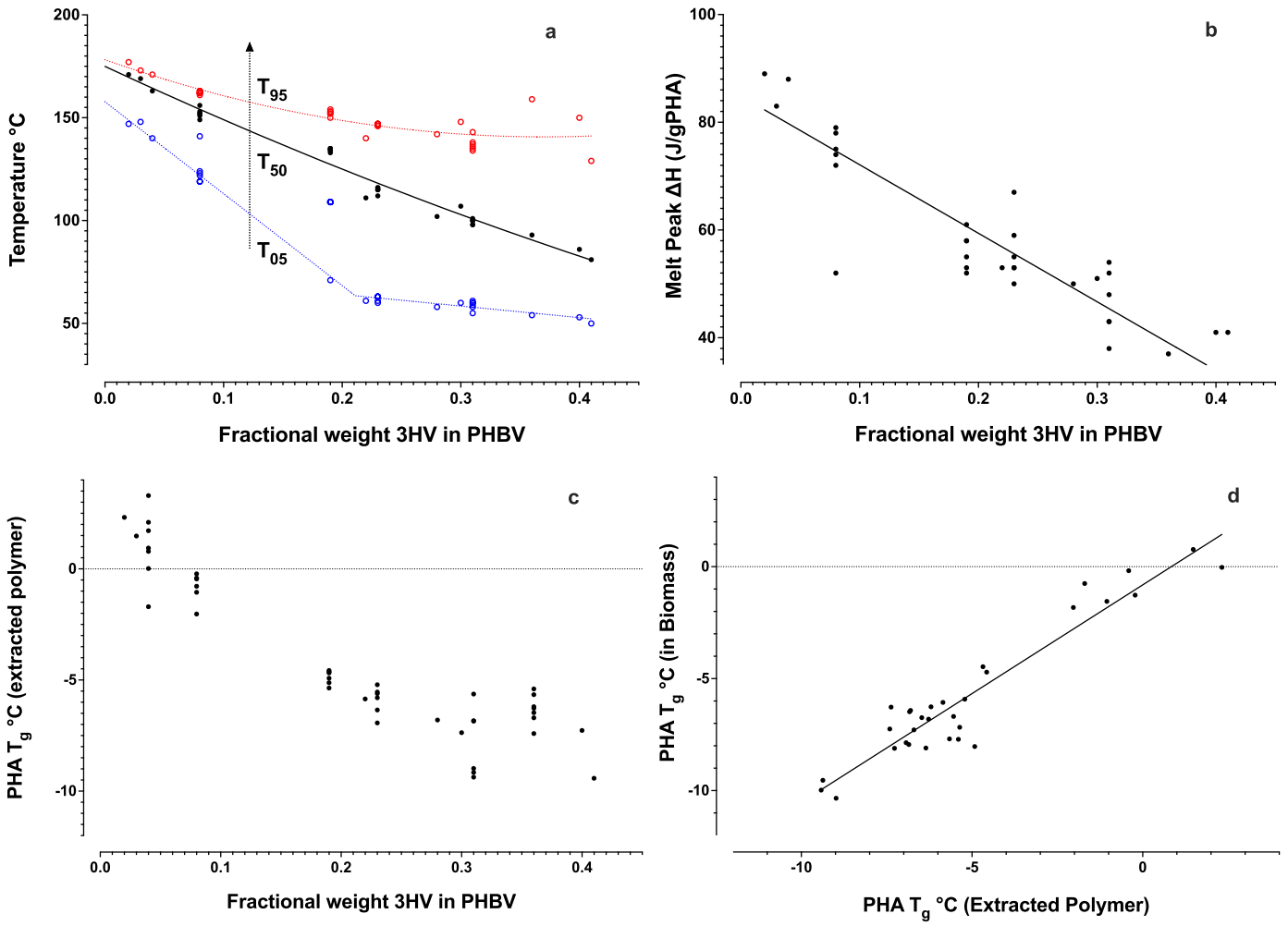

Fig. 11. Trend of melt temperatures and enthalpies for aged PHBVs as a function of average 3HV content (a and b). Estimated glass transition temperatures (T_g) for the same polymers (c). Comparison of the extracted polymer glass temperature versus the same polymer glass transition temperature measured from DSC of the biomass before extraction (d).

before PHBV extraction were not found to result in any systematic changes to melting temperatures as a function of 3HV content.

Melt enthalpies, for the aged polymer samples, were variable but followed an expected average trend of decreasing enthalpy with increasing average 3HV content due to decreased average crystallinity (Fig. 11b). The observed variability with respect to the trend line (Fig. 11b) was not correlated to the polymer average molecular weights or the total extract purities.

Glass transition temperatures were estimated for the extracted polymer and for the polymer *in-situ* in the dried biomass (Figs. 11c and d). The reported glass transition temperature was measured after quenching from 185 °C at −100 °C/min to −70 °C. T_g variability was not found to be correlated to the film purity (0.82 to 0.97 gPHA/g). An influence of molecular weight on T_g was expected based on previous work [18] and from the reviewed literature [4]. An influence of MW on T_g can result due to heat treatment related changes in the MW and 3HV distributions. For example, lower molecular weight PHB is reported to promote nucleation and microstructural developments influencing mechanical properties [66]. How the microstructure develops during quenching can influence if fewer or more 3HV units become excluded from crystalline regions in the microstructure [4]. However, T_g values, following MW decreasing trends for the same extracted polymer after pre-extraction heat treatments, did not follow a consistent trend in all cases to suggest a generic effect of molecular weight influence on thermal properties for these extracted films. Expressed variability of melt enthalpies and glass transition temperatures for any one batch of heat-treated biomass could indicate influences that were specific to the nature of respective blend changes in crystallization properties as a function of 3HV content and/or its distribution.

Good correspondence was found between estimated T_g values for the extracted polymer versus the polymer in the dried biomass (Fig. 11d). In the dried biomass, the PHBV will be finely distributed as distinct particles originating from the accumulated intracellular granules as present before dewatering and drying the biomass [67]. Each particle will represent the PHBV type and quality outcome for each respective individual accumulating microorganism. Distinctly different populations of particulate polymer type, linked to the species diversity in the mixed microbial community, can exhibit correspondingly different glass transition temperatures. The DSC measurement obtains an average signal from all the individual granules. Extraction is effectively a compounding step of blending all PHA granules, from all the species of microorganisms in the biomass, into a uniformly mixed solution. The extracted and dried polymer films in this work are a blended mixture from all contributing individual granules for each biomass sample. For the film samples, DSC measures an average signal due to the interactions generated by all polymer types that were distributed and blended from biomass. Thus, T_g measured in the biomass and for an extracted film from that biomass are different kinds of measurements and this may contribute to differences in Fig. 11d. An immiscible blend of polymer types may exhibit more than one glass transition temperature. For these samples, only one glass

transition temperature could be resolved in all cases.

The polymer average 3HV content will influence crystallinity. Thus, the average 3HV content is also correlated to T_g (Fig. 11c). Crystallinity influences extraction temperature for PHA-poor solvents (Fig. 1 and [35]). Therefore, DSC measurements for T_g in the dried biomass, and/or for T_m in dried biomass-solvent mixtures, can be applied for simple direct batch-to-batch screening for tuning of extraction conditions in industrial practice. Methods for quick, ideally *in-situ*, screening of PHA quality in the biomass before industrial scale extraction, is anticipated to become important for product quality control with batch-to-batch adjustments in the steps and conditions, including any pre-treatments, for optimal and consistent extraction (time, temperature, loading) with quality control.

4. Conclusions

A workflow has been developed for characterizing thermal treatment pre-processing of PHA-rich biomass before solvent extraction. Dimethyl carbonate (DMC) is a suitable solvent for generic routine evaluation of extractable PHA so long as sufficiently high extraction temperatures are applied. For PHB at least 125 °C is recommended. Extraction melting and crystallization gelation behaviour of PHAs in DMC and in other solvents can be systematically evaluated in suitable closed crucibles by DSC. DMC is also suitable for intrinsic viscosity measurements of PHAs including PHB. Intrinsic viscosity of a PHBV co-polymer blend (36 wt.% 3HV) in DMC at 60 °C correlated to weight average molecular weight (referenced to polystyrene) according to estimated Mark-Houwink constants α and LogK of 0.738 ± 0.010 and −2.016 ± 0.025, respectively.

A model of random scission with centre weighted molecule cleavage most closely represented the observations of molecular weight loss from practical experiments and GPC molecular weight distribution changes. The model simulations also supported that a scission number and scission rate (n_{sw} and r_{sw}) estimated specifically from weight average molecular weight (M_w) was a robust metric from which to follow and estimate cleavage kinetics in PHBV molecular weight loss.

Thermal pre-treatment before extraction generates reproducible outcomes of controlled molecular weight reduction. The rates and fate of M_w could be defined through the workflow and practical evaluations. Interpreted scission rates under isothermal conditions were commonly, but not always, constant. Measured properties of the polymer in the biomass (thermal stability, PHA content, PHBV grade, moisture content, etc.) could not be used to predict the variability in scission rates observed between the PHA-rich biomass batches that were evaluated. The sensitivity of the Mark-Houwink constants to PHBV type for intrinsic viscosity measured in DMC requires further investigation.

Molecular weight loss before extraction did not significantly influence the melting temperatures of the evaluated co-polymer blends of PHBVs over the wide sub-eutectic range of average 3HV content that was tested. Variability in observed glass transition temperatures or melting enthalpies for a heat-treated biomass were not correlated to average molecular weight loss. The variability in melt enthalpies and glass transition temperatures was considered to nevertheless be influenced by the molecular weight loss. Molecular weight loss can result in changes to PHBV blend 3HV distributions with anticipated resulting effects on the polymer crystallization behaviour.

CRediT authorship contribution statement

Alan Werker: Conceptualization, Methodology, Software, Investigation, Formal analysis, Visualization, Supervision, Writing – original draft, Project administration. **Ruizhe Pei:** Conceptualization, Methodology, Investigation, Formal analysis, Validation, Visualization, Supervision, Writing – review & editing. **Kevin Kim:** Methodology, Investigation, Formal analysis, Visualization. **Giulia Moretto:** Methodology, Investigation, Formal analysis, Visualization. **Angel Estevez-Alonso:** Methodology, Investigation, Validation, Supervision, Writing – review & editing. **Chris Vermeer:** Investigation, Validation. **Monica Arcos-Hernandez:** Investigation, Validation. **Jelmer Dijkstra:** Methodology, Validation, Visualization, Supervision, Writing – review & editing. **Erik de Vries:** Investigation, Supervision, Project administration.

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.

Data availability

Data will be made available on request.

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