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Green solvent extraction and properties characterization of Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) biosynthesized by mixed microbial consortia fed fermented dairy manure

Maryam Abbasi [a], Erik R. Coats [b], Armando G. McDonald [a,*]

[a] Department of Forest, Rangeland and Fire Sciences, University of Idaho, Moscow, ID 83844-1133, USA
[b] Department of Civil and Environmental Engineering, University of Idaho, Moscow, ID, USA

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ABSTRACT

The aims of this study were to assess the large-scale extraction and recovery of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) biosynthesized by mixed microbial consortia (MMC) that was fed fermented dairy manure at the pilot level using green solvents (ethanol, cyclohexanone (CYC), and dimethyl carbonate (DMC)). The reflux extraction procedure with CYC for 3 h at 130 °C and DMC for 4 h at 90 °C showed the highest recovery (32.3%) with an overall pure yield of 30.0%. A modified Soxhlet extraction process was also evaluated and gave lower crude (18–20%) and purified (12.7–13.8%) PHBV yields. The molar mass of extracted PHBV with DMC in reflux was 7.07×10^5 g·mol^{-1} which was comparable to the control. The thermal, mechanical, and rheological behavior of extracted PHBV were also examined. Mechanical properties result showed that strength and modulus values of the polymers extracted in the large Soxhlet extractor were lower than those extracted by reflux.

1. Introduction

Due to their versatility, petroleum-derived polymers have played a significant role in modern day life. However, the high rate of plastic waste generation and the resistance to biodegradation of petrochemical based plastics has caused severe environmental problems. A variety of methods for disposal of petrochemical based plastics have been used including landfilling, incineration, and even just depositing in the environment. Almost 80% of plastic wastes are disposed of in landfills. This creates an increasing pile of plastic wastes and their distribution on the planet has made considerable environmental pollution (Koller, 2019). Depositing plastic wastes into the oceans causes harm to marine and endangers human health through the introduction of microplastic particles (< 5 mm) into the food cycle (Koller, 2019; Campanale et al., 2020). The annual global production of petrochemical plastics was 330 megatons (Mt) in 2019, from which 150 Mt. of plastic waste enters the oceans (Koller, 2019). Disposing of plastic waste in landfills ends with contamination of terrestrial and aquatic environments, through their migration via the wind or water systems, into the oceans (Koller, 2019; Worm et al., 2017; Guo et al., 2020). While incineration of plastic wastes can be used for energy production it has led to the formation and release of greenhouse and toxic gaseous emissions (Koller, 2019). The recycling of petrochemical based plastics is limited by consideration of the required purity for the plastic waste recovered and due to quality and material fatigue (Koller, 2019). The depletion of fossil fuel sources and the consequences of poor degradability of the petrochemical plastics has increased the demand to replace them with sustainable bio-derived and biodegradable plastics like polyhydroxyalkanoates (PHA) (Zheng et al., 2020).

PHAs are biodegradable polyesters that can be produced through microbial biosynthesis on renewable feedstocks. PHAs accumulate intracellularly as an energy and carbon storage under stressed growth conditions in the presence of excess carbon source (Yu and Chen, 2006; Koller et al., 2013; Choi and Lee, 1999; Kim et al., 2003). PHAs, and specifically the most known copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), show properties similar to conventional petrochemical-based synthetic plastics and after disposal are entirely biodegraded to water and carbon dioxide by microorganisms (Choi and Lee, 1999; Koller et al., 2011). However, the widespread production of PHBV bioplastics is hampered by their high production cost, including the upstream and downstream recovery after fermentation, compared to commodity petroleum-derived plastics (Zheng et al., 2020; Yu and Chen, 2006; Koller et al., 2013; Choi and Lee, 1999). It has been reported using mixed microbial cultures (MMCs), which do not require a sterile

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[*] Corresponding author at: Department of Forest, Rangeland and Fire Sciences, University of Idaho, 875 Perimeter Drive MS 1133, Moscow, ID 83844-1133, USA.
E-mail address: armandm@uidaho.edu (A.G. McDonald).

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environment, and the potential of broader metabolism compared to single strain has provided the opportunity to use a wide variety of low-cost carbon sources (Samorì et al., 2015a). A variety of natural feedstocks including municipal and industrial wastewater (Dionisi et al., 2004; Amanat et al., 2021; Lorini et al., 2021), vegetable oils (Moralejo-Gárate et al., 2014; Campanari et al., 2017), agricultural and forestry wastes (Guho et al., 2020; Amanat et al., 2021; Yin et al., 2020;, and food industry wastes (Colombo et al., 2016; Hilliou et al., 2016; Morgan-Sagastume et al., 2020) have been used as main nutritional substrate.

The downstream processing, including pre-treatment, extraction, and purification of the PHBV biopolymer, covers more than 50% of the total PHBV production cost (Guo et al., 2020; Samorì et al., 2015a; Haddadi et al., 2019; Pérez-Rivero et al., 2019; López-Abelairas et al., 2015). Two general strategies have been adopted for the separation of PHBV granules from cellular biomass: PHBV solubilization, and cellular biomass dissolution (Yu and Chen, 2006; Samorì et al., 2015b). It is reported that the direct extraction of PHBV granules from biomass serves as the best method (Koller et al., 2013). A variety of methods for PHBV extraction based on the two aforementioned strategies have been studied including solvent extraction, extraction using chelate- surfactant, chemical extraction, biological extraction, enzymatic extraction and mechanical disruption (Haddadi et al., 2019; Jacquel et al., 2008). Among different PHBV extraction methods, the solvent extraction method, especially halogenated solvents, including chloroform ($CHCl_3$), methylene chloride, and 1,2-dichloroethane, have been widely studied (Guho et al., 2020; Pérez-Rivero et al., 2019; Ramsay et al., 1994). Halogenated solvent extraction provides the extracted polymer with high purity and low endotoxin content without polymer degradation. However, it provides the extracted polymer with low recovery, is harmful to the environment, is not economical, and also affects the natural morphology of the polymer, thereby limiting the use of PHA in specific applications like durable fibers production (Pérez-Rivero et al., 2019; Kynadi and Suchithra, 2014; Jacquel et al., 2008). In view of the adverse effects of halogenated solvents, researchers have now focused on green solvents for PHBV recovery such as esters, alcohols, amides, cyclic and acyclic ketones. Many successful small laboratory-scale studies have been performed on PHA extraction with green solvents like dimethyl carbonate (DMC), cyclohexanone (CYC), methanol, anisole, and ionic liquids (Rosengart et al., 2015; Samorì et al., 2015b). Solvent extraction can provide the polymer with a high purity, but the high volume of required solvent, high required temperature, and potential human and environment toxicity are the hindrance through commercializing this method. While the process of extraction with surfactants and chelating agents is simple, it needs a high surfactant dose which increases recovery cost, requires large volume of water, and provides the polymer with low purity and significantly reduced molar mass (Righi et al., 2017; Jacquel et al., 2008; Haddadi et al., 2019; Samorì et al., 2015a). Chemical extraction methods yield polymers with a high molar mass but with a low recovery and purity. In comparison, relatively inexpensive biological extraction methods result in polymers with a high molar mass but low yield and endotoxin materials as by-products. However, the biological materials involved in this extraction need to be pretreated. Mechanical and enzymatic extraction methods are environmentally friendly and have low contamination, however they are expensive and complex processes (Haddadi et al., 2019; Bhattacharyya et al., 2012). Unfortunately, PHBV extraction studies have been performed at small scale ($\leq$ 3 g of biomass) and there is a gap in studies using larger-scale extractions for commercialization and comprehensive characterization of extracted PHBV (Samorì et al., 2015b; García et al., 2019; Rosengart et al., 2015).

The aim of this study was to evaluate green solvents for the extraction of PHBV in small and moderate scale from PHBV rich biomass obtained from a pilot-scale bioprocess using MMC fed fermented dairy manure (Guho et al., 2020). The Glaxo Smith Kline (GSK) solvent sustainability guide helps scientists in an objective assessment of using solvents (Alder et al., 2016). Various factors including reactivity and stability, environmental impact, environmental health, and life cycle score of different solvents are considered (Pérez-Rivero et al., 2019; Henderson et al., 2011; Alder et al., 2016). Considering the GSK solvent guide, green solvents having a score > 5 in environmental impact, safety and health, and the literature (García et al., 2019; Samorì et al., 2015[b]; Rosengart et al., 2015) were used to select a suitable extraction solvent for PHA. Specifically, the green solvents evaluated were DMC, CYC, and ethanol, with $CHCl_3$ as control for PHBV yield and recovery using the reflux extraction method. A modified Soxhlet extraction method at small and large-scale was also evaluated. PHBV composition, molar mass, thermal, mechanical, and rheological properties of the extracted PHBVs were determined to establish whether extraction/purification influenced their properties.

2. Materials and methods

2.1. Materials

CYC ($\geq$ 99%) and DMC (99%) were obtained from Alfa Aesar (Ward Hill, MA, USA). Ethanol (EtOH, 99%) and acetone (99%) were technical grade. $CHCl_3$ (99.8%) was from EMD Millipore (Burlington, MA, USA).

PHBV rich biomass was produced in a pilot-scale operation consisting of (i) sequencing batch dairy manure fermenter (760 L) to produce volatile fatty acids (VFA), (ii) an enrichment reactor (680 L) which was fed VFAs to produce a MMC culture that can produce PHBV via a feast-famine regime and (iii) PHBV production reactor (450 L) which was fed VFAs to produce and accumulate PHBV and reported in detail in a previous study (Guho et al., 2020). The lyophilized PHBV rich biomass (PHBV content of 31.1% (*w*/w); HV molar ratio 0.29 ± 0.03) was used for the subsequent extraction studies in reflux and small Soxhlet extractor. The lyophilized PHBV rich biomass (PHBV content of 24.5%–35.6% (w/w); and HV molar ratio 0.26 ± 0.00 to 0.32 ± 0.00) was used for the extraction studies in large Soxhlet extractor. The operational conditions such as solvent type (EtOH, DMC, CYC, and $CHCl_3$), temperature (58–130 °C), contact time (30–300 min), and extraction steps (1 to 3 times) in reflux, small and large Soxhlet extractor were studied.

2.2. PHBV solvent extractions

2.2.1. Small scale reflux method

Lyophilized biomass (20 g) was washed with acetone (400 mL, 3×) for 24 h to remove lipids, filtered and air dried. The lipid-free biomass was extracted with either EtOH, DMC, CYC, and $CHCl_3$ in reflux with constant stirring to obtain crude PHBV. Table 1 gives extraction process conditions used and yields. A polymer/solvent <5% (*w*/*v*) was required to have a suitable viscosity to process effectively (Choi and Lee, 1999). In the 1st step 300–600 mL solvent was used. In the 2nd and 3rd steps 250–400 mL and 150 mL of solvent were used, respectively. The extracts were hot filtered (P8 filter paper, Fisher Scientific) in a heated bath to remove cell debris. Extracts from steps 1–3 were combined, evaporated to dryness (rotary evaportator) under vacuum (60 °C) to a constant weight and the crude polymer yield recorded. The purification process on crude PHBVs was variable depending on the solvent used for extraction. The purification process for PHBV's extracted with each solvent is given below.

The crude PHBV from $CHCl_3$ extraction was dissolved in $CHCl_3$ and then added dropwise to cold petroleum ether (700 mL), under constant stirring, and PHBV recovered as a precipitate. The precipitated PHBV was further dissolved in $CHCl_3$ and added dropwise to cold methanol (700 mL), under constant stirring, and pure PHBV recovered as a white precipitate, vacuum dried and yield recorded. The crude PHBV's from DMC and CYC extraction were dissolved in $CHCl_3$ and added dropwise to cold methanol (700–1200 mL) under constant stirring. The precipitated PHBV was vacuum dried (60 °C), and yield recorded. The crude PHBV from DMC extraction only required one purification step, while the CYC required two methanol purification steps.

Table 1
Operational condition, yields, and purity of PHBV extraction with dimethyl carbonate (DMC), cyclohexanone (CYC), ethanol (EtOH) and $CHCl_3$ control on lipid-free biomass in reflux, small and medium scale Soxhlet extractor.

Sample	Solvent	Apparatus	Extraction steps	temperature	Time	PHBV Yield (%)		Purity
				(°C)	(h)	Crude	Pure	(%)
$CHCl_3$	$CHCl_3$	Reflux	2	58–60	3,1	30.6 ± 2.2	23.1 ± 0.0	94.9 ± 2.8
CYC-1	CYC	Reflux	1	120	1	27.1	22.88	87.0 ± 2.8
CYC-2	CYC	Reflux	3	120	1,1,1	27.2	26.36	99.3 ± 0.8
CYC-3	CYC	Reflux	2	130	2,1	35.5 ± 0.1	32.3 ± 1.0	98.2 ± 1.0
CYC-4	CYC	Reflux	2	130	3,1	32.8 ± 0.3	30.6 ± 1.0	96.3 ± 2.5
DMC-1	DMC	Reflux	2	90	3,1	33.5 ± 0.8	30.0 ± 1.0	98.4 ± 1.2
DMC-2	DMC	Reflux	2	90	4,1	30.1 ± 0.1	25.4 ± 2.4	96.4 ± 1.6
DMC-3	DMC	Reflux	2	90	1,1,	31.1	26.59	95.4 ± 1.2
EtOH -1	EtOH	Reflux	1	77	0.5	3.7	–	–
EtOH -2	EtOH	Reflux	1	77	1	3.5	–	–
EtOH -3	EtOH	Reflux	1	77	5	8.9	–	–
DMC-S1	DMC	small Soxhlet	1	40	4	16.6	13.3	99.0 ± 2.6
DMC-S2	DMC	small Soxhlet	1	40	24	17	14	98.9 ± 1.1
DMC-S3	DMC	modified small Soxhlet	1	60–75	4	20.8	18.5	96.4 ± 2.5
$CHCl_3$-S4	$CHCl_3$	small Soxhlet	1	40	4	19.1	14.2	99.4 ± 1.7
$CHCl_3$-S5	$CHCl_3$	small Soxhlet	1	40	24	36.8	29.7	99.9 ± 1.2
DMC-S6	DMC	modified large Soxhlet	1	60–75	11	19	12.7	97.2 ± 3.5
DMC-S7	DMC	modified large Soxhlet	1	60–75	12	18.2	12.7	96.1 ± 1.5
DMC-S8	DMC	modified large Soxhlet	1	60–75	14	20	13.8	94.0 ± 4.8

The PHBV extraction with ethanol was performed in three different treatments (Table 1). Lyophilized biomass (21 g) was suspended in EtOH (700 mL), homogenized for 1–4 min, and then refluxed (77 °C). The EtOH extract was filtered to recover the biomass, acetone was added (350–700 mL) to the filtrate, and PHBV was recovered after centrifugation (7200 rpm, −10 °C, for 10 min), vacuum dried, and yield recorded.

Crude PHBV yields were based on ash free lyophilized biomass weight basis. Pure PHBV was calculated from GCMS analysis according to the literature (Guho et al., 2020; Braunegg et al., 1978).

2.2.2. Small scale Soxhlet method

Acetone washed biomass samples (≤5 g) were Soxhlet extracted with either DMC or $CHCl_3$ (150 mL) for 4 and 24 h (Table 1). In extraction with DMC, a modified Soxhlet extraction process was also employed to improve extraction efficiency by heating the extraction unit thimble zone to 60–75 °C using heating tape. The extracts were rotary evaporated, dried in a vacuum oven (60 °C) to record the crude yield and purified with the same method explained for PHBV extracted with DMC and $CHCl_3$ in the reflux, respectively and yields recorded.

2.2.3. Large scale Soxhlet method

The lyophilized PHBV rich biomass (415 to 680 g) (S6; PHBV content of 24.5% (w/w); HV molar ratio 0.27 ± 0.00, S7; PHBV content of 35.6% (w/w); HV molar ratio 0.32 ± 0.00, and S8; PHBV content of 32.7% (w/w); HV molar ratio 0.26 ± 0.00) were used for the extraction in the large Soxhlet extractor. Acetone washed biomass (391 to 649 g) was placed in a polypropylene filter bag and Soxhlet extracted with DMC (5–7 L) for 11–12 h using a modified extractor in which the extraction bag zone was heated to 60–75 °C with heating tape (Table 1). The purification steps were the same as the DMC extracted material in reflux (4.5–7 L methanol).

2.3. Analytical methods

PHBV composition was performed on purified PHBV (1 mg), in triplicate, by methanolysis (3% H_2SO_4 in methanol (2 mL) and $CHCl_3$ (2 mL, containing 0.25 $mg{\cdot}mL^{-1}$ benzoic acid as internal standard)) in a digestion test tube and the mixture heated at 100 °C for 4 h. The 3HV/3HB ratio as their methyl ester derivatives were analyzed by GC–MS (Trace1300-ISQ7000, Thermo Scientific) on a ZB-1 capillary column (30 m × 0.25 mm, Phenomenex, Torrance, CA USA) with a temperature profile of 40 °C (1 min) ramped to 200 °C (10 min) at 5 $°C{\cdot}min^{-1}$ (Guho et al., 2020).

The number (M_n) and weight average (M_w) molar mass and the polydispersity index of PHBV samples (2 $mg{\cdot}mL^{-1}$) were determined, in duplicate, by size exclusion chromatography (SEC) multi-angle light scattering (MALS) analysis. Separation was achieved using a Jordi DVB linear mixed bed column (7.8 mm × 300 mm) on elution with $CHCl_3$ (0.5 $mL{\cdot}min^{-1}$) coupled to a refractive index (Waters model 2414, Milford, MA, USA) and MALS (PostNova Analytics PN3609, 658 nm, Landsberg, Germany) detectors. A refractive index increment (DN/DC) value of 0.034 for PHB in $CHCl_3$ was used (Guho et al., 2020). Data were analyzed using the NovaSEC v1.5.0 software. Calibration of the system was performed using a narrow polystyrene standard (Malvern, M_w = 105,268 $g{\cdot}mol^{-1}$).

^{1}H-NMR spectra of PHBV in $CDCl_3$ were recorded on a 300 MHz Bruker spectrometer. Spectra were analyzed using TopSpin V3.6.2 software. The ^{1}H-NMR spectra obtained for 3HV and 3HB were used for determination of 3HV molar fraction through the ratio of the integrated ^{1}H peak areas of the 3HV methyl resonance and the sum of 3HB + HV methyl resonance (Wei et al., 2014; Guho et al., 2020).

FTIR spectra of PHBV samples were obtained using a Nicolet-iS5 spectrometer (Thermo-Scientific, Madison, WI, USA), 64 scans, with an attenuated total reflection (ATR) probe (ZnSe crystal). Spectra were baseline corrected and averaged using the Omnic v9.0 software. To determine carbonyl index ($I_{C=O}$), which reflects the crystallinity of PHBV biopolymers, the normalized spectra were curve-fitted using Igor Pro 9 software (WaveMetrics) (Wei and McDonald, 2015; Wei et al., 2015a, 2015b). The area (A) of each band gained from curve fitting was integrated by the software. The $I_{C=O}$ was calculated as the ratio of the areas (A) under carbonyl (C=O) bands in 1720–1740 cm^{-1} of PHBV ($I_{C=O} = A_{1720}/A_{1740}$).

Differential scanning calorimetry (DSC) experiments on PHBV samples (5 mg) were performed using a Q200 DSC (TA Instruments, New Castle, DE, USA) with refrigerated cooling 40 °C (3 min) then ramped to 180 °C (3 min) at 10 $°C{\cdot}min^{-1}$ and subsequently cooled to −50 °C (3 min) at −10 $°C{\cdot}min^{-1}$, and the heating-cooling cycles repeated. The glass transition (T_g), melting (T_m) and crystallization temperatures (T_c), and the degree of crystallinity (X_c%) were obtained from the peak maximum of the exotherm and endotherm of the second heating/cooling scan. The X_c% of PHBV samples was calculated using the melting enthalpy of 100% crystallized PHBV at 146 $J{\cdot}g^{-1}$ (Wei et al., 2014). Data were analyzed using TA Universal analysis v4.5A software.

The thermal stability of PHBV was determined by thermogravimetric analysis (TGA) on a Perkin Elmer TGA-7 instrument (Shelton, CT, USA)

(5 mg, from 30 °C to 800 °C at 20 $°C \cdot min^{-1}$ under nitrogen (30 $mL \cdot min^{-1}$)). Data was analyzed using the Pyris v13.3 software.

The rheological behavior of PHBV (25 mm Ø × 1.2 mm) was studied using a parallel plate rheometer (Bohlin CVO 100 NF, NJ, USA) from 0.01 to 100 Hz (0.0628–628 $rad \cdot s^{-1}$), 0.5% strain, and 180 °C. Data were analyzed (power-law models) using the Bohlin rheology v6.51.0.3 and Microsoft Excel software.

PHBV (0.3 g) samples were hot pressed at 160–180 °C in a preheated die-press mold (Ø 25 mm) to obtain 0.5 mm thick discs. Samples were cut into strips (10 × 2.5 × 0.5 mm^3). Tensile (gauge length 10 mm) and three-point bending (10 mm span length) tests were each performed at room temperature on a DMA Q800 (TA instruments, New Castle, DE, USA) with controlled force (3 $N \cdot m^{-1}$) and constant strain ($1\% \cdot min^{-1}$) until yield, respectively. Tensile strength, Young's modulus, elongation to break, flexural strength, and flexural modulus were determined from the obtained stress-strain curve using Universal Analysis V4.5A software. At least five test specimens were measured per sample.

3. Result and discussion

3.1. PHBV yields

PHBV rich biomass was produced at pilot scale by MMC fed fermented dairy manure (Guho et al., 2020). The removal of lipid extractable material from the lyophilized biomass was achieved using acetone (5–7% of the biomass weight). PHBV was then extracted on the acetone washed biomass. The yields of crude and pure PHBV from the reflux method are given in Table 1. Among the polymers extracted by reflux, PHBV extracted with CYC-3, CYC-4 and DMC-1 methods showed the highest crude yield (35.5%, 32.8% and 33.5%) and the highest pure yield (32.3%, 30.6% and 30.0%), respectively. These crude yields were slightly higher than the crude yield of PHBV extracted with $CHCl_3$ (30.6%) by reflux. Also, the pure PHBV yield extracted with CYC and DMC, except CYC-1 (25.4%–32.3%), was higher than the pure PHBV yield with $CHCl_3$ (23.1%). The crude yields of DMC extracted PHBV were higher than those reported in the literature (crude yield 20%, and purity 98% for 60 min, at 90 °C, without pretreatment) (Samorì et al., 2015b).

While EtOH was introduced as a promising green solvent for PHA extraction and shows good pure yield and purity in lab-scale (82% and 82%, respectively) (García et al., 2019), the crude yield in this study was low (3–9%). Due to the low crude yield, experiments with EtOH were not continued. The composition (3HV molar fraction) of the reflux EtOH extracted PHBVs were determined by GC–MS (3HV = 0.22 to 0.28) and ^1H NMR spectroscopy (3HV = 0.21 to 0.25) (Table 2). These extracted PHBV samples have 3HV molar fraction values that were comparable to the cellular biomass (~0.29).

The reflux extraction yields were influenced by extraction time and temperature. Extraction with DMC was at a lower temperature (90 °C) than CYC (120–130 °C) and similar extraction time (240 min) but resulted in similar yields. By increasing the extraction temperature from 120 to 130 °C for CYC a 50% improvement in yield was obtained; this is consistent with previous observations (Fiorese et al., 2009). Increasing the extraction time from 60 to 180 min for CYC did not improve crude

Table 2
3HV composition, molar mass, and thermal properties of the various purified PHBV samples obtained from different extraction methods.

Solvent/ Sample name	3HV molar fraction[b]		Molar Mass[b]			Thermal properties[b]								
	Purified PHBV		M_w	M_n	PDI[a]			DSC			FTIR		TGA	
						T_g	T_{m1}	T_{m2}	T_c	X_c	$I_{C=O}$	T_{onset}	T_d	T_{endset}
	GC–MS (mol. mol^{-1})	^1H NMR (mol. mol^{-1})	($\times 10^5$ $g \cdot mol^{-1}$)	($\times 10^5$ $g \cdot mol^{-1}$)		(°C)	(°C)	(°C)	(°C)	(%)		(°C)	(°C)	(°C)
$CHCl_3$	0.25 ± 0.01	0.24 ± 0.01	7.18 ± 0.22	6.35 ± 0.44	1.1 ± 0.0	–7.9 ± 0.4	NA	161.9 ± 0.2	71.2 ± 1.5	19.7	2.35	259 ± 5	291 ± 4	298 ± 4
CYC-1	0.22 ± 0.01	0.21 ± 0.01	3.09 ± 0.35	2.94 ± 0.28	1.05 ± 0.02	–7.1 ± 0.5	147.7 ± 0.2	163.3 ± 0.1	80.9 ± 0.6	19.6	2.20	283 ± 0	310 ± 1	317 ± 1
CYC-2	0.23 ± 0.00	0.22 ± 0.01	3.22 ± 0.12	2.88 ± 0.07	1.12 ± 0.02	–7.0 ± 0.2	148.8 ± 0.4	163.7 ± 0.1	73.5 ± 0.3	21.2	2.38	295 ± 1	321 ± 1	327 ± 1
CYC-3	0.24 ± 0.01	0.24 ± 0.00	2.54 ± 0.19	2.35 ± 0.16	1.08 ± 0.01	–7.0 ± 0.5	151.6 ± 2.7	164.7 ± 0.9	86.4 ± 10.4	19.1	2.28	287 ± 2	314 ± 2	319 ± 2
CYC-4	0.24 ± 0.00	0.23 ± 0.01	2.03 ± 0.16	1.82 ± 0.22	1.12 ± 0.06	–7.2 ± 0.1	149.5 ± 0.3	164.0 ± 0.2	81.34 ± 0.3	20.1	2.29	293 ± 2	317 ± 3	323 ± 3
DMC-1	0.25 ± 0.00	0.25 ± 0.00	7.08 ± 0.53	5.91 ± 0.34	1.20 ± 0.03	–7.4 ± 0.2	153.9 ± 0.5	162.4 ± 1.5	82.5 ± 2.3	17.8	2.24	283 ± 0	314 ± 2	319 ± 2
DMC-2	0.28 ± 0.00	0.24 ± 0.01	6.45 ± 0.32	5.15 ± 0.33	1.26 ± 0.1	–7.7 ± 0.8	153.1 ± 0.5	162.0 ± 1.8	84.1 ± 7.7	18	2.14	285 ± 4	315 ± 3	321 ± 3
DMC-3	0.28 ± 0.00	0.24 ± 0.00	6.27 ± 0.30	5.39 ± 0.15	1.16 ± 0.02	–7.5 ± 0.4	NA	161.4 ± 0.4	78.5 ± 1.0	18.5	2.36	285 ± 3	316 ± 2	323 ± 3
DMC-S1	0.33 ± 0.00	0.31 ± 0.00	5.7 ± 0.2	4.3 ± 0.1	1.33 ± 0.02	–5.9 ± 0.4	NA	156.9 ± 0.7	51.3 ± 0.4	1.4	–	273 ± 1	304 ± 0	310 ± 1
DMC-S2	0.36 ± 0.00	0.35 ± 0.00	5.4 ± 0.1	4.3 ± 0.1	1.26 ± 0.01	–5.3 ± 0.2	NA	157.2 ± 0.4	49.2 ± 0.3	1.3	–	266 ± 2	300 ± 1	306 ± 0
DMC-S3	0.27 ± 0.01	0.26 ± 0.02	6.4 ± 0.1	4.8 ± 0.2	1.35 ± 0.04	–7.9 ± 0.2	NA	162.0 ± 0.5	75.2 ± 0.8	16	–	264 ± 0	299 ± 1	305 ± 2
$CHCl_3$-S4	0.25 ± 0.00	0.24 ± 0.01	7.4 ± 0.1	5.7 ± 0.1	1.3 ± 0.02	–8.6 ± 0.5	NA	157.8 ± 0.4	97.9 ± 0.3	17.9	–	262 ± 1	294 ± 1	301 ± 1
$CHCl_3$-S5	0.25 ± 0.01	0.25 ± 0.01	8.5 ± 0.9	7.3 ± 0.9	1.16 ± 0.01	–6.9 ± 0.5	156.1 ± 0.3	165.6 ± 0.1	95.4 ± 0.0	18	–	264 ± 0	298 ± 2	303 ± 1
DMC-S6	0.27 ± 0.00	0.25 ± 0.01	22.5 ± 1.6	21.9 ± 1.5	1.03 ± 0.01	–4.0 ± 0.1	NA	164.5 ± 04	45.8 ± 1.1	5.60	2.06	288 ± 2	310 ± 2	317 ± 1
DMC-S7	0.30 ± 0.00	0.29 ± 0.01	8.6 ± 0.3	8.0 ± 0.3	1.07 ± 0.01	–6.0 ± 0.2	NA	165.7 ± 0.2	77.7 ± 0.2	13.8	2.04	285 ± 3	310 ± 1	317 ± 2
DMC-S8	0.30 ± 0.00	0.28 ± 0.02	9.8 ± 0.2	9.2 ± 0.1	1.06 ± 0.01	–5.8 ± 0.6	NA	162.5 ± 0.1	46.7 ± 0.5	5.7	2.22	284 ± 1	310 ± 2	317 ± 1

[a] PDI = M_w / M_n.
[b] Values are mean ± standard deviation.

PHBV yield, but did increase pure PHBV yield and purity; this observation is consistent with others (Fiorese et al., 2009). The reduction of PHBV yield at longer extraction times may be attributed to the degradation of polymer chains (Rosengart et al., 2015; Hahn et al., 1994; Fiorese et al., 2009).

Issues observed with using CYC were (i) its strong odor, even working in the fume hood, (ii) the hot filtration step raised safety concerns, and (iii) an additional purification step required to obtain reasonable purity, and therefore using CYC in subsequent trials was not further pursued. Due to the lengthy process of PHA recovery using the reflux method, a more efficient/streamlined extraction process, Soxhlet extraction, was investigated. The crude and pure PHBV yields for the Soxhlet extraction process are given in Table 1. Using a small Soxhlet extractor, with $CHCl_3$ as a control for 24 h, the crude PHBV yield was 37% (pure PHBV yield 30%). These results were comparable to those of polyhydroxybutyrate (PHB) extracted with $CHCl_3$ from single strains (recovery 31%, and purity 92% at 37 °C (Valappil et al., 2007), and recovery 27%, and purity 95% at 25 °C (Ramsay et al., 1994)). However, using DMC as solvent the PHBV yields were low (~17%), likely due to the extraction temperature around 30 °C in the thimble. The Soxhlet extraction unit was then modified to heat the extraction thimble chamber to about 60–75 °C and this resulted in a crude PHBV yield increase to 21% (pure PHBV yields 14–18%). Increasing the extraction time from 4 to 24 h also improved PHBV yields. This result is in agreement with the literature (crude yield 17% and purity 87%) (Samorì et al., 2015a). The PHBV 3HV molar ratios (determined by GC–MS) using the small Soxhlet unit were between 0.25 and 0.36 (Table 2).

Due to the ease of using the Soxhlet apparatus with DMC, a larger extractor with a heated extraction chamber was employed to extract PHBV from the biomass. Crude and purified PHBV yields of 18–20% and 13–14% were obtained, respectively (Table 1). The low yields could be attributed to the lower extraction temperature (60–75 °C) as compared to the reflux method (90 °C). Further work is required to optimize yields and will be part of a future study. The 3HV molar ratios were between 0.27 and 0.3 (Table 2). The Soxhlet extraction method was simpler to operate, DMC (80%) could be readily recovered, without the need for hot filtration, however the yields were lower.

3.2. PHBV molar mass determination

The molar mass (M_n and M_w) of extracted and purified PHBV samples was determined by SEC-MALS and the results are given in Table 2. The M_w and M_n values for the $CHCl_3$ reflux extracted PHBV control were 7.18×10^5 and 6.35×10^5 g·mol^{-1}, respectively; the PHBV M_w was 6–9% lower than observed by Wei et al. using PHBV-rich biomass obtained from a bench-scale bioreactor fed fermented dairy manure (Wei et al., 2014). The DMC reflux extracted PHBV samples were comparable (M_w 6.3–7.1 $\times 10^5$ g·mol^{-1} and M_n 5.2–5.9 $\times 10^5$ g·mol^{-1}) to that of the control. In contrast the CYC reflux extracted PHBV had lower M_w (2.0–3.2 $\times 10^5$ g·mol^{-1}) and M_n (1.8–2.9 $\times 10^5$ g·mol^{-1}) values. The lower M_w and M_n for the CYC extracted PHBV is likely due to hydrolysis at 120–130 °C and this phenomenon has been reported (Rosengart et al., 2015; Fiorese et al., 2009). The PDI for the reflux extracted PHBV samples ranged between 1.05 and 1.26.

The M_w of the Soxhlet (small and large) extracted PHBV samples was between 5.4 and 14.3 $\times 10^5$ g·mol^{-1} and in the range obtained using the reflux extraction method. The large Soxhlet extracted PHBV with DMC had the lowest PDI (1.01–1.07), while the PDI values for PHBV extracted with the small Soxhlet extractor were higher (1.16–1.35). The PHBV sample DMC-S6 had a significantly higher M_w and M_n than other extracted PHBV samples by Soxhlet extraction, which cannot be explained. According to the literature, continuous extraction with green solvents such as carbonates, esters, and ketones can recover polymers with high molecular weight (1.1–1.4 $\times 10^6$ g·mol^{-1}) and constant PDI (1.4–3.9) at lab-scale (Pagliano et al., 2021).

3.3. FTIR spectral analysis

FTIR spectroscopy was used to analyze the chemical features of the various purified PHBV samples extracted with either CYC, DMC, and $CHCl_3$. Overall, the spectra for all the PHBV samples were similar. The bands at 1720 cm^{-1} with a shoulder at 1740 cm^{-1} having the highest intensity belong to carbonyl (C=O) stretching, which corresponded to crystalline and amorphous components of PHBV, respectively (Guho et al., 2020; Bai et al., 2015; Wei et al., 2015a, 2015b; Xu et al., 2021). The band at 1275 cm^{-1} was assigned to saturated ester linkage (C—O stretch) (Pramanik et al., 2014; Bai et al., 2015), while the band vibration at 977 cm^{-1} presents the C—C stretching vibration (Aguiar et al., 2018). A band at 1178 cm^{-1} was also present and assigned to C–O–C stretching in an amorphous structure (Zhou et al., 2019). The bands between 1054 and 1129 cm^{-1} and 1226 cm^{-1} were assigned to the C—O stretching (Bai et al., 2015). The four bands at 1378 ($-CH_3$), 1456 ($-CH_3$), 2933 ($-CH_2$), and 2975 cm^{-1} ($-CH_3$) are attributed to C—H bending vibrations (Bai et al., 2015; Pramanik et al., 2014; Aguiar et al., 2018).

The carbonyl index ($I_{C=O}$) of the PHBV biopolymers was calculated from the ratios of fitted bands of crystalline PHBV regions at 1720 cm^{-1} to amorphous PHBV regions at 1740 cm^{-1} (Table 2) (Wei and McDonald, 2015; Wei et al., 2015a, 2015b). All the samples extracted with different solvents by reflux ($CHCl_3$, CYC-1 to CYC-4, and DMC-1 to DMC-3) showed similar $I_{C=O}$ which ranged between 2.14 and 2.38, while the samples DMC-S6 to DMC-S8 extracted using the large Soxhlet showed lower $I_{C=O}$ values (1.97–2.04). These results agree with X_c by DSC for PHBV extracted with various solvents in reflux and with DMC using the large Soxhlet (18–21% by reflux and 5.6–13.8% by Soxhlet) (Wei and McDonald, 2015; Wei et al., 2015a, 2015b).

3.4. Thermal analysis of PHBV

The thermal properties (T_g, T_m, T_c and X_c) of purified PHBV samples were determined by DSC. The second heating and cooling DSC thermograms are shown in Fig. 1. The T_g, T_m, and X_c values were determined from the second heating cycle, while the T_c was determined on the second cooling cycle (Table 2). The results show that PHBV extracted with CYC, DMC-1, DMC-2 and $CHCl_3$-S5 have two melting points – a low-temperature melting peak (T_{m1}, 147 to 156 °C) and a high-temperature melting peak (T_{m2}, 156 to 165 °C). The T_g values from PHBV extracted by reflux with DMC, CYC, and $CHCl_3$ were similar (−7 to −8 °C), while the T_g from the small Soxhlet extractor with DMC and $CHCl_3$ was −5 to −8 °C. PHBV extracted with DMC using the large Soxhlet unit had a T_g from −4 to −6 °C. The extraction method had no considerable influence on the T_m values and were comparable with published studies (Koller et al., 2013). In a separate study, PHB extracted with $CHCl_3$ had a T_m of 173–176 °C while PHB extracted with EtOH had a T_m of 179 °C (García et al., 2019). This result shows the improvement effect of HV monomer in widening the processing window of extracted PHBV comparing PHB (Shang et al., 2012).

The presence of two melting peaks could be attributed to the isodimorphism phenomena during the heating of PHBV (Guho et al., 2020; Bluhm et al., 1986; Lv et al., 2016). The isodimorphism phenomena in semi-crystalline PHBV with some higher HV content crystals present will have a higher amorphous phase ratio and thus will be melted (T_{m1}) first. The lower HV content crystals present having a higher crystallinity ratio require more energy to melt and thus melt at a higher temperature (T_{m2}) (Souza Junior et al., 2019; Gahlawat and Soni, 2017; Hasan et al., 2018). Other factors that can lead to two melting points include differences in crystalline morphology (thickness, perfection, lamellar stability, or distribution), relaxation of the rigid amorphous phase, physical aging, and presence of crystallites with different molar masses (Souza Junior et al., 2019; Laycock et al., 2014). The data also suggests that the X_c of the extracted PHBV by reflux were similar (17%–21%), resulting in similar mechanical properties. The X_c of the polymers extracted with DMC using the small Soxhlet extractor (DMC—S1, and —S2) was <3%,

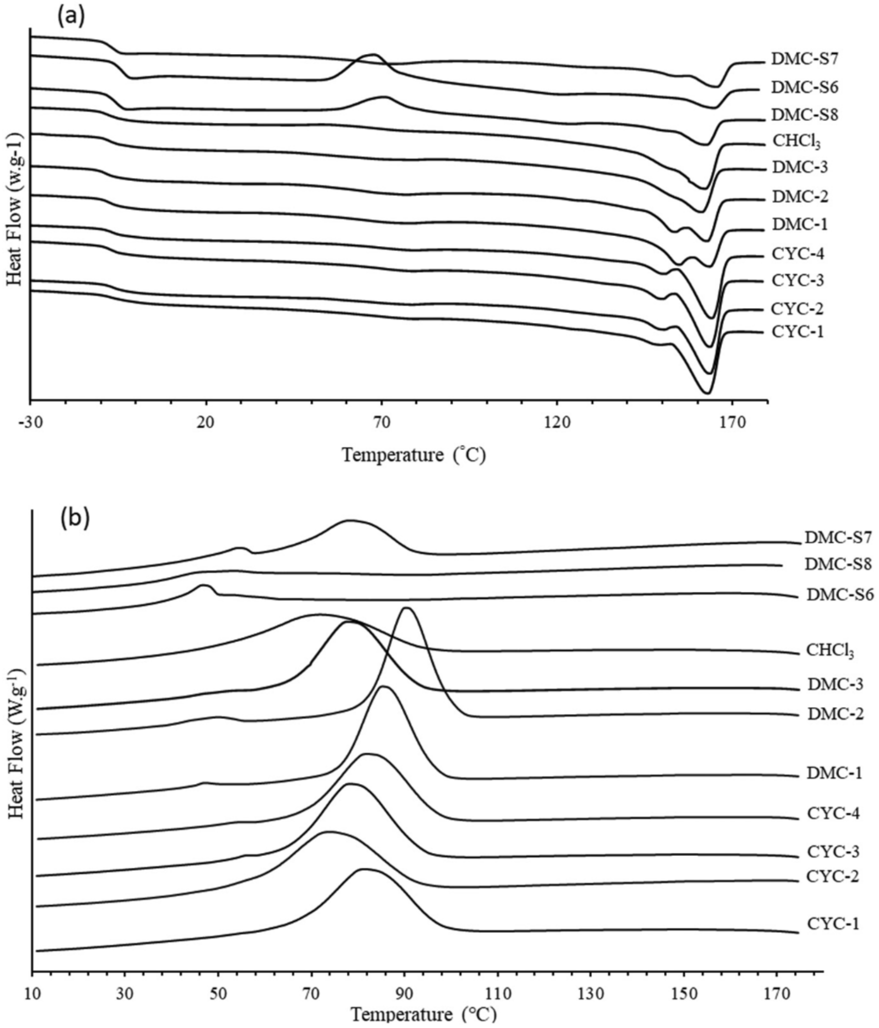

Fig. 1. DSC thermograms of (a) the second heating cycle and (b) the second cooling cycle of the various purified PHBV samples.

which was significantly lower than the crystallinity of PHBV with similar HV% extracted with $CHCl_3$ (Dai et al., 2007; Serafim et al., 2008). The crystallinity of 34% has been reported for PHBV with 35% HV extracted with $CHCl_3$ (Dai et al., 2007). In another study, for PHBV extracted with $CHCl_3$ having the HV monomer content of 4.6% the X_c of 17.8% and for PHBV with 72% HV content the X_c of 6.7% is reported (Serafim et al., 2008). However, PHBV extracted using the heated Soxhlet system (DMC—S3) showed higher X_c (16%) which is close to that obtained with $CHCl_3$ (~18%). The X_c of PHBV extracted with DMC using the large Soxhlet extractor with heating (DMC—S6, —S7, and —S8) was significantly lower (6–14%) than that obtained using the small Soxhlet extractor (16%). This is likely due to the higher HV content of these polymers (0.27–0.3).

The $I_{C=O}$, from FTIR spectroscopy, of all the samples extracted with different solvents in reflux ($CHCl_3$, CYC-1 to CYC-4, and DMC-1 to DMC-3) ranged between 2.14 and 2.38, while the samples DMC-S6 to DMC-S8 extracted using the large Soxhlet showed lower $I_{C=O}$ values (1.97–2.04). A poor correlation ($R^2 = 0.43$) between $I_{C=O}$ and X_c by DSC for the extracted PHBVs was observed. Published work on PHBV extracted using different methods showed X_c values between 54.4% and 64.1% and $I_{C=O}$ values between 0.74 and 0.78, with samples having higher X_c exhibiting higher $I_{C=O}$ (Valappil et al., 2007). In another study, the $I_{C=O}$ for $CHCl_3$ extracted PHBV (0–47% HV) ranged between 0.81 and 0.99, and crystallinity determined by X-ray diffraction (62 to 69%) showed that the $I_{C=O}$ values were nearly independent of HV content (Bloembergen et al., 1986). The X_c of PHBV can be decreased from 40 to 2.5% by cross-linking with dicumyl peroxide, with a similar decrease in $I_{C=O}$ from 1.3 to 0.39 (Wei et al., 2015a, 2015b; Wei and McDonald, 2015).

The T_c for the reflux extracted PHBV ranged between 71 and 86 °C (Table 2). PHBV extracted with $CHCl_3$ using the small Soxhlet extractor showed the highest T_c (95–98 °C) vs. PHBV extracted using other extraction conditions. PHBV extracted with DMC using the Soxhlet (DMC—S1, —S2, —S6, and —S8) had the lowest X_c (1–6%) and T_c (45–51 °C). The PHBV samples DMC-S3 and —S7 showed similar T_c (75–77 °C), which was slightly lower than PHBV extracted with DMC under reflux (78–84 °C). Suhazsini et al. obtained a T_c value of 108.9 °C for PHBV (16.6% HV) extracted with NaClO and $CHCl_3$ (Suhazsini et al., 2020). Melendez-Rodriguez et al. observed a T_c between 41 and 82 °C for $CHCl_3$ extracted PHBV with varying HV contents (20–60%) (Melendez-Rodriguez et al., 2021). In another study, the T_c for PHBV (20% HV) fiber and film cultured using fruit pulp biowaste was 65.6 and 71.2 °C (Melendez-Rodriguez et al., 2018), respectively. The presence of an additional exothermic peak in the heating cycle of the DMC-S6 and —S8 implies that the nucleation and crystal formation were slower for these samples compared to the others (Fig. 1a) (Chotchindakun et al., 2021). A reduction in T_c corresponds to a reduced melting enthalpy, meaning a lower X_c (Chotchindakun et al., 2021).

The thermal degradation of PHBV was performed by TGA. The thermograms of purified PHBV samples showed a significant 1-step degradation due to a nonradical random chain scission mechanism comprising a six-membered ring transition state (Da Silva Moreira Thiré et al., 2011; Chen et al., 2013; Liu et al., 2009). The thermal onset

(T_{onset}), thermal end set (T_{endset}), and degradation temperature (T_d) of the purified PHBV samples are given in Table 2. PHBV extracted with $CHCl_3$ in reflux had a significantly lower T_{onset} (259 °C) vs. the polymers extracted using different conditions. Also, T_{onset}, T_{endset}, and T_d were lower in PHBV extracted with DMC (DMC—S1, —S2, and —S3) and $CHCl_3$ ($CHCl_3$ —S4, and —S5) using the small Soxhlet extractor than other PHBV extracted with different solvents in reflux and with DMC in large scale Soxhlet extractor.

T_{onset} of the PHBV extracted with DMC and $CHCl_3$ in the small Soxhlet extractor ranged between 262 and 273 °C, while T_d ranged from 294 to 304 °C and T_{endset} ranged from 301 to 310 °C. For PHBV extracted with CYC and DMC in reflux and large scale Soxhlet extractor, the T_{onset}, T_d, and T_{endset} were 283–295 °C, 310–321 °C, and 317–327 °C, respectively. The highest reported T_d for natural P3HB is 290 °C (Ramsay et al., 1994). The thermal stability (as assessed by T_d) of PHBV is attributed to the HV content of PHB (Carrasco et al., 2006). Weight loss of PHBV CYC-1 started at lower temperature than the other polymers which is possibly due to the polymer being still embedded in the proteinaceous cellular residue and the higher impurity compared with extracted material (Samorì et al., 2015b). The TGA thermograms of most PHBV samples showed at >95% weight loss and associated with thermal degradation (Shang et al., 2012). The large difference between T_m (by DSC) and T_d (by TGA) for PHBV provides the processability window for this material and thus makes them suitable for molding and extrusion for marketable products (Liu et al., 2019; García et al., 2019; Koller et al., 2013).

3.5. Rheology

Rheology provides information on how to process (e.g., extrusion) polymer melts by measuring the flow (viscosity) behavior as a function of shear rate (Bhat and Kandagor, 2014). The complex viscosity (η^*) of the PHBV samples extracted using the reflux and Soxhlet methods at 180 °C versus shear rate (ω), also known as flow curves, are shown in Fig. 2. The PHBV flow curves show a reduction in η^* with ω which is indicative of shear thinning behavior typical for non-Newtonian fluid (Liao et al., 2009). At high shear rate the anisotropic polymer chains with high molar mass will disentangle and align and thus less molecular interaction among polymer chains occurs resulting in a higher free volume which leads to a reduction in viscosity (Modi et al., 2013; Liao et al., 2009).

The η^* of PHBV sample CYC-1 was shown to decrease more rapidly with ω than the other PHBV samples. This might be ascribed to the lower purity (87%) of the polymer and partial plasticizing effect of impurities present in the polymer (Hilliou et al., 2016). The M_W of the polymers extracted with DMC by reflux and the large Soxhlet extractor and $CHCl_3$ by reflux was considerably higher than the M_W of the polymers extracted with CYC by reflux. In addition, their zero-shear viscosity was lower than the polymers extracted with CYC by reflux, which can be attributed to the simultaneous effect of higher crystallinity which increases the η^* and lower M_w which decreases the η^* in polymers extracted with CYC (Lim and Hoag, 2013).

To assess the shear thinning behavior quantitatively, the rheological data were fitted to the Power–law (Ostwald–de Waele) model, representing η^* as a function proportional to some power of the ω (Modi et al., 2016). The relationship between steady shear and dynamic shear viscosity has been established using the Cox–Merz rule, which is applicable for PHA, PHB, and PHBV (Modi et al., 2016). A modified Power-law model was obtained by exchanging the steady shear terms with dynamic viscosity terms that is expressed as:

$$|\eta^*(\omega)| = K(\omega)^{n-1} \tag{1}$$

where, K is the consistency coefficient and n is the non-Newtonian or flow behavior index. These parameters were determined from the double log plots of η^* vs. ω (Fig. 2). For $n < 1$ the material is pseudo-plastic, when $n = 1$ the material is Newtonian, and $n > 1$ the material is dilatant (Emadzadeh et al., 2015; Gupta, 2005; Modi et al., 2016). However, one drawback of this model is that at the low and high shear regions the power law model deviates considerably from the experimental data and should not be extended to these ranges (Emadzadeh et al., 2015; Gupta, 2005). The goodness of fit for the models R^2 was used. All the PHBV samples had $R^2 > 0.96$. The K and n parameters, and η^* at $\omega = 6.28$ $rad \cdot s^{-1}$ for the various PHBV samples are given in Table 3. The Power–law model fit the data well, with K values ranging from 3.39 to 31.2 Pa.s. The n values ranged from 0.738 to 0.850 showing pseudo-plastic behavior. Values from this study were considerably different to those reported in the literature for PHBV (5% HV content) at 160 °C ($K = 1.35 \times 10^6$ Pa.s and $n = 0.0823$) (Modi et al., 2016; Modi et al., 2013). Differences in measurement temperature (160 vs. 180 °C) may be a contributing factor in the different K values.

For comparative purposes, the η^* of the PHBV samples at $\omega = 6.28$

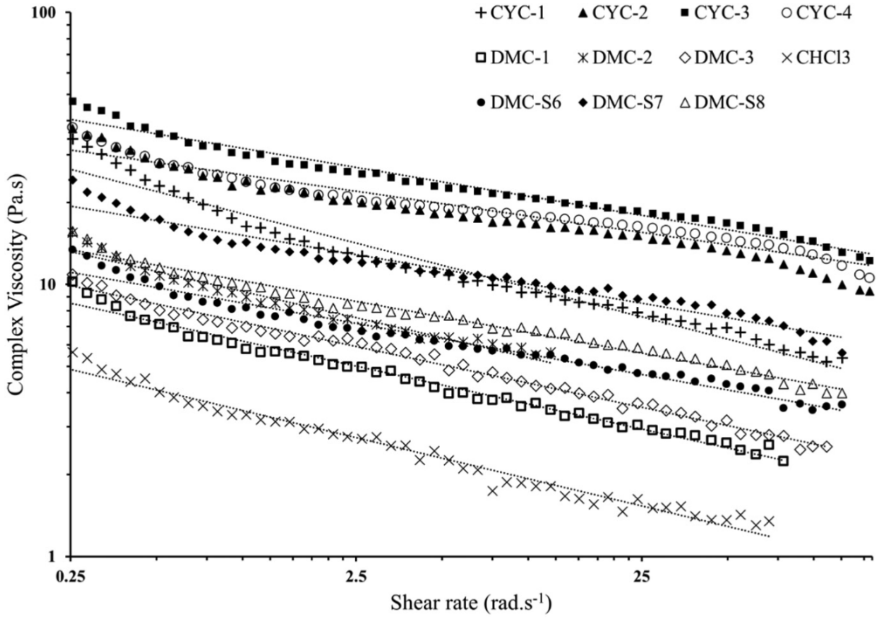

Fig. 2. Complex viscosity (η^*) versus shear rate (ω) plots for the various extracted PHBV samples (180 °C) and power-law fitted models (dotted lines).

Table 3
Power–Law parameters (K and *n*) and complex viscosity (η^*) at $\omega = 6.28$ rad·s^{-1} (1 Hz) determined at 180 °C for the various PHBV samples.

Solvent/Sample name	K (Pa.s)	*n*	η^* (Pa.s)
CYC-1	17.7	0.738	10.95
CYC-2	24.6	0.829	17.97
CYC-3	31.2	0.829	27.77
CYC-4	25.0	0.850	18.94
DMC-1	6.09	0.776	4.03
DMC-2	9.26	0.771	6.08
DMC-3	7.20	0.779	4.8
$CHCl_3$	3.39	0.756	2.16
DMC-S6	8.38	0.819	6.01
DMC-S7	14.9	0.829	10.84
DMC-S8	10.2	0.815	7.23

rad·s^{-1} = 1 Hz was determined and ranged from 2.16 Pa.s to 27.8 Pa.s. The polymers extracted with CYC showed a higher η^* than the other samples ranging from 11.0 Pa.s to 27.8 Pa.s. The PHBV extracted with $CHCl_3$ by reflux showed the lowest η^* at 2.16 Pa.s. The η^* of the PHBV samples extracted with DMC in reflux and large Soxhlet, except for DMC—S7, were similar (4.03–7.23 Pa.s). The η^* of DMC-S7 sample was similar to CYC-1 sample. The difference in the η^* may be attributed to the difference in HV content of the samples. The lower η^* is associated with PHBV of higher HV content.

3.6. *Mechanical properties*

The mechanical properties (tensile and flexural) of the various extracted PHBV samples were determined and the results are given in Table 4. The tensile strength of the polymers extracted with various solvents in reflux ranged between 6.5 MPa and 12.4 MPa and the Young's modulus of the samples ranged between 0.25 GPa and 0.86 GPa. The tensile strength values of 29.1 MPa for solution cast PHBV (5% HV) (Thiagamani et al., 2019), 27.1 MPa for injection molded PHBV (8% HV) (Jiang et al., 2008a), and 14.1 MPa for solution cast PHBV (12% HV) (Jiang et al., 2008b) have been reported. Furthermore, the Young's modulus of 2.19 GPa for solution cast PHBV (5% HV) (Thiagamani et al., 2019), 3.5 GPa for injection molded PHBV (8% HV) (Jiang et al., 2008a), and 0.82 GPa for solution cast PHBV (12%) HV (Jiang et al., 2008b) have also been documented. Strength and modulus values were inversely related to HV content. The Young's modulus of PHBV from the large Soxhlet extractions (DMC—S6, —S7, and —S8) ranged from 0.25 to 0.4 GPa and were lower than PHBV extracted by the reflux method (0.41–0.86 GPa).

The flexural strength of large Soxhlet extracted PHBV (DMC—S6, —S7, and -S8; 7.3 to 9.3 MPa) was lower than the reflux extracted PHBV (14.8–18.6 MPa), while the flexural modulus large Soxhlet extracted (DMC—S6, —S7, and -S8; 0.31 to 0.41 GPa) was lower than the reflux extracted samples (0.56–0.9 GPa). Flexural strength and modulus values of compression molded PHBV (28.2 MPa and 1.28 GPa) (Persico et al., 2011) and injection molded 8% HV PHBV (34.5 MPa and 3.2 GPa) (Jiang et al., 2008a) have been published, respectively. Again, strength and modulus values were inversely related to HV content (Lim and Hoag, 2013).

Table 4
Tensile and flexural properties of the various extracted PHBV samples[1,2].

Solvent/ Sample	Tensile strength	Young's modulus	Flexural strength	Flexural modulus
	MPa	GPa	MPa	GPa
$CHCl_3$	9.5 ± 0.6^{b}	0.47 ± 0.02^{bc}	17.0 ± 0.6^{ab}	0.73 ± 0.07^{bc}
CYC-1	6.5 ± 1.3^{b}	0.86 ± 0.17^{a}	16.8 ± 0.5^{abc}	0.90 ± 0.07^{a}
CYC-2	12.4 ± 1.1^{b}	0.61 ± 0.08^{b}	18.6 ± 1.1^{a}	0.77 ± 0.15^{abc}
CYC-3	10.3 ± 1.3^{b}	0.56 ± 0.08^{b}	17.1 ± 0.8^{ab}	0.71 ± 0.04^{bc}
CYC-4	11.8 ± 0.8^{b}	0.59 ± 0.05^{b}	17.5 ± 0.7^{a}	0.78 ± 0.06^{b}
DMC-1	11.2 ± 1.3^{b}	0.52 ± 0.07^{bc}	15.5 ± 0.5^{bc}	0.67 ± 0.05^{cd}
DMC-2	9.9 ± 0.7^{b}	0.51 ± 0.03^{bc}	17.2 ± 0.4^{a}	0.74 ± 0.02^{bc}
DMC-3	10.0 ± 1.6^{b}	0.41 ± 0.06^{c}	14.8 ± 0.4^{c}	0.56 ± 0.05^{d}
DMC-S6	8.8 ± 0.6^{b}	0.40 ± 0.04^{a}	9.3 ± 0.9^{d}	0.41 ± 0.04^{e}
DMC-S7	8.0 ± 0.8^{a}	0.30 ± 0.06^{a}	7.3 ± 0.7^{d}	0.31 ± 0.03^{e}
DMC-S8	9.6 ± 0.3^{b}	0.25 ± 0.03^{a}	7.3 ± 0.5^{d}	0.32 ± 0.02^{e}

[1] Values are mean ± standard deviation.

[2] Statistical differences of means using a Tukey test (*P*-value <0.05) and different superscript letters (a, b, c, d, e) shows significance.

3.7. *Relationships between PHBV properties*

The parameters of solvent type, extraction time and temperature in two different extraction apparatus were studied here. Changes in these parameters effected on the yields and HV content of the extracted PHBV. The effect of extraction methods on yields is discussed earlier. Comparing methods, PHBVs extracted in small and large Soxhlet had a higher range of HV content. This can be because of the lower temperature of the solvent in contact with biomass in Soxhlet than reflux which provides a heating energy that is enough for extraction of mostly amorphous high HV content structure than crystallized high HB content structure. By increasing the extraction time to optimum the HV content and M_W of the extracted PHBVs increased. When extraction time exceeded the optimum level, the HV content and the M_W of the extracted PHBVs decreased. The previous studies have shown that the longer extraction time can result in the hydrolysis of the polymer chains. Because polymer granules decompose rapidly, the molecular weight reduction can be minimized by shortening extraction time (Mohammadi et al., 2012; Choi and Lee, 1999).

Generally, regardless of extraction method the HV content of the extracted polymers influenced the M_W, PDI, X_c and $I_{C=O}$ values. Thermal properties including T_m, T_c, and T_g decreased with HV content. This can be attributed to the fact that an increase in the HV content of the polymer increases the amorphous structure and reduces the nucleation of PHBV and initiates crystallization at a lower temperature (Chotchindakun et al., 2021).

The η^* of PHBVs extracted with CYC (CYC-1 to –4) and DMC (DMC-1 to- 3 and DMC-6 to –8) increased with HV content.

The mechanical properties of PHBV showed a negative relationship with HV content ($R^2 \geq 0.63$) which is due to the higher amorphous content (Lim and Hoag, 2013).

4. Conclusions

Protocols for green solvent (CYC, DMC, and EtOH) extraction of PHBV from fermented manure by MMC was successful. DMC was a suitable solvent for PHBV extraction with >80% recovery, and one purification step from the crude extract but lower PHBV yields. Modified Soxhlet extraction used exhibited suitable extraction, ease of use, and workup. Molar mass, polydispersity, and mechanical properties were comparable for PHBV extracted with DMC and $CHCl_3$ (control) but higher melt viscosity (η^*) for the former. DMC solvent was efficiently used on a large lab-scale extraction but needs further research optimization to improve yields for greener industrial extraction processes.

CRediT authorship contribution statement

Maryam Abbasi: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Writing.

Erik R. Coats: Supervision; Funding acquisition; Writing.

Armando G. McDonald: Supervision; Project administration; Funding acquisition; Writing; Methodology; Conceptualization.

Declaration of competing interest.

The authors declare that they have no known financial or personal conflicts that might influence the work in this article.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2022.101065.

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