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Development of a rapid method to isolate polyhydroxyalkanoates from bacteria for screening studies

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Abstract

We describe a novel method of Polyhydroxyalkanoate (PHA) extraction using dimethyl sulphoxide (DMSO) for use in screening studies. Compared to conventional chloroform extraction, the DMSO method was shown to release comparable quantities of PHA from *Cupriavidus necator* cells, with comparable properties as determined using FT-IR spectroscopy and differential scanning calorimetry.

Main text

Polyhydroxyalkanoates (PHAs) are a class of ubiquitous biological polymers generated in a range of organisms during times of carbon excess and utilised during carbon starvation (1). They are typified by poly-3-hydroxybutyrate (P(3HB)), a PHA generated by many bacteria such as *Cupriavidus necator* from sugars or waste streams (2). Bacterially-derived PHAs have been identified as potentially useful biological polymers for replacement of petrochemically-derived plastics due to their non-reliance on crude oil for production and their biodegradability. However, P(3HB) undergoes secondary crystallisation following processing leading to poor polymer properties (chiefly progressive embrittlement (3)) and so many researchers are currently developing novel PHA polymers with enhanced properties.

A second major problem faced in the development of cost-effective commercial PHAs is isolation and purification (4). Conventional techniques are costly, representing up to 50% of the overall cost of PHA (5). Many use halogenated solvents such as chloroform or dichloromethane to disrupt lysophilised bacteria and solubilise PHA; at laboratory scale, this is usually done under reflux in a Soxhlet apparatus using a relatively large quantity of chloroform (typically 30 mL per 300 mg of dry biomass). The PHA dissolved in chloroform is then precipitated using a second solvent such as hexane or ethanol. The whole process is labour- and time-intensive, requires lysophilisation of bacteria and high solvent use. Alternative approaches to PHA extraction were reviewed by Jacquel *et* al. (4); recent approaches published in the literature include use of detergents (6), protease treatment (7) and alkaline treatment (5). However, development of alternative PHA extraction techniques has not been investigated as extensively as the development of PHA polymers with improved properties.

In this study, we investigated the use of dimethyl sulphoxide (DMSO) as a nontoxic solvent for the extraction of PHA from *C. necator* cells. DMSO is an aprotic solvent (it does not establish hydrogen bonds) which is also miscible with polar solvents as it possesses a dipole moment. It is able to dissolve lipophillic molecules such as PHA and can readily pass across biological membranes, including those present in Gram positive bacterial cell walls. These properties make DMSO a potential solvent for extraction of PHA from bacteria. The method was optimised using flow cytometry and the resultant P(3HB) tested against P(3HB) extracted by conventional methods (chloroform reflux) using FTIR and DSC.

C. necator strain H16 (DSM428; DSMZ, Braunschweig, Germany) was grown in MSM medium using fructose as a carbon and energy source at a C:N ratio of 30:1 g/g, conditions under which poly-3-hydroxybutyrate is generated. MSM contained 2.3 gL^{-1} KH_2PO_4, 2.9 gL^{-1} $Na_2HPO_4 \cdot 2H_2O$, 1 gL^{-1} NH_4Cl, 0.5 gL^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 gL^{-1} $CaCl_2 \cdot 2H_2O$, 0.05 gL^{-1} Fe(NH_4) citrate and 5 mL trace element solution SL-6 (comprising 0.1 gL^{-1} $ZnSO_4 \cdot 7H_2O$, 0.03 gL^{-1} $MnCl_2 \cdot 4H_2O$, 0.3 gL^{-1} H_3BO_3, 0.2 gL^{-1} $CoCl_2 \cdot 6\ H_2O$, 0.01 gL^{-1} $CuCl_2 \cdot 2H_2O$, 0.02 gL^{-1} $NiCl_2 \cdot 6H_2O$ and 0.03 gL^{-1} $Na_2MoO_4 \cdot 2H_2O$). Precultures were prepared in 250 mL conical flasks containing 20 mL of ME medium (5 gL^{-1} Peptone and 3 gL^{-1} Meat extract) inoculated with a loopful of *C. necator* and incubated for 24 h at 30 ^{o}C and 200 rpm. Two litre conical flasks containing 200 mL of MSM were inoculated with a volume of this preculture required to result in an optical density at 600 nm (OD_{600}) of 0.1. Five millilitres of 40 % (w/v) fructose solution was added to each culture after 24 and 48 hours. After 72 h growth, cultures were harvested by centrifugation and resuspended in phosphate buffered saline (PBS).

Addition of DMSO to *C. necator* H16 cell suspensions was found to rapidly clear the suspension, presumably by bacterial lysis. Conversely, DMSO did not lyse *C. necator* PHA^{-}4 cells which cannot generate P(3HB). This suggested that DMSO enters the *C. necator* cells

and interacts with P(3HB): cells containing P(3HB) lysed, releasing the P(3HB) into solution, whereas those without P(3HB) did not lyse.

The lysis of *C. necator* by DMSO was investigated using flow cytometry (BD Accuri C6 flow cytometer, BD Biosciences, Oxford, UK). *C. necator* H16 was grown as previously described. Bacteria were stained with 1 μgml^{-1} Pyrromethene 546 (Exciton, Ohio, USA; a 0.1 $mgmL^{-1}$ stock solution in 10% DMSO), a lipophillic dye that enters bacteria and stains PHA green, and flow cytometry was used to determine the PHA accumulation of individual bacteria within the culture (Fig. 1a). As is frequently observed, there was a great deal of hetereogeneity within the culture and not all bacteria generated PHA. In contrast, *C. necator* PHA$^-$4 cells (strain DSM541), grown under the same conditions, were shown not to accumulate PHA at all due to a deletion in the genes encoding PHA production (data not shown).

C. necator H16 were resuspended in PBS at a concentration of 74.6 mg dry biomass mL^{-1}. Aliquots (250 μL) of this cell suspension were added to 50 mL of DMSO incubated at 70 °C with agitation. The OD_{600} of the DMSO and cell suspension mixture was measured after the addition of each aliquot of cell suspension (Fig. 1b). The measured OD_{600} of the DMSO and cell suspension mixture was far lower than expected, suggesting that the majority of cells lysed upon addition to DMSO. Further successive 250 μL aliquots of cell suspension were added to the DMSO every 5 minutes. Flow cytometry was used to analyse the DMSO – cell suspension mixture during successive addition of cell suspension. Samples were doubly stained with Pyrromethane 546 and 0.4 μM SYTO62 (Invitrogen; a DNA dye that stains all cells red). After addition of the equivalent of 380 mg of dry cells, flow cytometry revealed one population of cells that had a low concentration of PHA as determined by pyrromethene 546 staining (Fig. 1c). This corresponds to *C. necator* cells that had not accumulated PHA; as

shown in Fig. 1a, a sub-population of cells fail to accumulate PHA in liquid culture. However, after addition of the equivalent of 450 mg dry cells, two populations were visible by flow cytometry (Fig. 1d): one comprising cells containing a low quantity of PHA; and one comprising cells containing more PHA, comparable to Fig. 1a. These populations were still present after an additional 1 hour of incubation at 70 °C (Fig. 1e). In addition, the gradient of the OD_{600} versus biomass added graph increased after addition of the equivalent of 380 mg dry biomass, indicating that cells were no longer being effectively lysed by the DMSO (Fig. 1b). Taken together, this indicates that 50 mL of DMSO could effectively lyse 380 mg of *C. necator* biomass containing P(3HB).

Following solubilisation of P(3HB) in DMSO, the P(3HB) was precipitated by addition of ethanol. Optimisation experiments using different ratios of DMSO to ethanol and different ethanol temperatures revealed that cold ethanol (-20 °C) precipitated P(3HB) more rapidly than ethanol at higher temperatures, and that a ratio of 3 volumes of ethanol to one volume of DMSO / P(3HB) mixture was optimal. Using these conditions, precipitation was complete after 160 minutes incubation at 4 °C. Following incubation, the ethanol and DMSO liquid phase was partially decanted and water was added (volume equivalent to that of DMSO initially used), which resulted in PHB aggregation. The PHB aggregate was collected by vacuum filtration with a Whatman 54 (22 μm) filter paper. The retentate was freeze-dried overnight prior to storage.

To benchmark the new DMSO method in comparison with Soxhlet extraction, *C. necator* DSM428 was cultured in 1 L baffled flasks containing 200 mL of MSM with 1% Fructose. This culture was harvested by centrifugation for 10 minutes at 5000 rpm in 2 fractions of 100 mL each. One fraction was extracted with the DMSO method using 20 mL of DMSO and 60 mL of ethanol, while the other was freeze-dried and extracted with a Soxhlet apparatus. Three hundred milligrams of freeze-dried cells were deposited in a Soxhlet thimble and 50 mL of

was chloroform used to extract PHA at 85 ^{o}C for 7 hours. The PHA was precipitated from the chloroform by addition to 150 mL of pre-cooled ethanol at -20 ^{o}C and vacuum filtered with Whatman #1 filter paper. The PHA precipitate was then freeze dried.

Yields from both methods were comparable: Soxhlet extraction yielded 2.51 mg of P(3HB) per mL of culture while DMSO extraction yielded 2.79 mg per mL of culture. FTIR was performed on solid pellets of P(3HB) which were placed directly under ATR (Thermo Nicolet 380 FTIR with Smart Orbit attached set to 'absorbance', a resolution of 1 cm^{-1} and 128 scans) for spectrum detection. In terms of peak locations, the FTIR spectrum of the DMSO extracted P(3HB) sample (Fig. 2a) was very similar to that of Soxhlet-extracted P(3HB) and commercial P(3HB) (Goodfellow Cambridge Ltd., Huntingdon, UK). General differences in peak intensities can be attributed to different clamping forces resulting from differing sample geometries and/or inhomogeneities. However, closer inspection of the spectra shows that a number of bands contain peaks in very similar locations, but with differing relative intensities. This may indicate differing crystalline morphologies (and degree of crystallinity).

P(3HB) extracted by DMSO and chloroform Soxhlet extraction were compared using differential scanning calorimetry using a Mettler Toledo DSC 1 (Mettler Toledo, Leicester, UK) calibrated with indium and tin standards. The P(3HB) samples (~5 mg) were weighed into a 40 μL aluminium DSC pan (Mettler Toledo) which was then capped with an aluminium DSC pan lid (Mettler Toledo) and sealed with a press (Mettler Toledo). The sample were subjected to the following programme, the temperature was held at -40 °C for 5 minutes before raising it to 200 °C at a rate of 10 °C min^{-1}. Following a 5 minute isotherm at 200 °C, the sample was cooled to -40 at a rate of 10 °C min^{-1}. This procedure was repeated twice in order to impose a known thermal history on the samples.

Each trace shows a broad melting peak in the region of 160 to 180 °C (figure 2b). On the first cycle it is worth noting that melting region of the DMSO extracted sample is composed of what appears to be multiple melting peaks. This suggests either multiple populations of crystal lamella thicknesses or that the sample is flowing during melting which is leading to variable thermal contact with the DSC cell. The shape of the main melting peak is consistent with the latter. On cooling, both samples re-crystallised, but in the case of the DMSO extracted sample, the re-crystallisation process occurred at a reduced temperature range. This observation suggests a difference in the crystallisation kinetics of the recovered polymers. In addition, the recrystallization process in cycle two (DMSO extracted) occurs at an elevated temperature range. This suggests that some residual order persists in the sample after the first melt. This is commonly observed in polymers where melting has taken place below the equilibrium melting temperature, which can exceed the observed melting temperature by a significant margin. In the case of PHB, heating to temperatures just above the observed melting point is prudent since the polymer is thermally unstable.

The thermal properties of P(3HB) extracted by the two methods are summarised in Table 1. On the first heating runs, the melting points of the two P(3HB) samples vary by 8 °C (DMSO 170 °C and chloroform 178 °C) however, on cooling and reheating, the melting points are virtually identical at c.173 °C. Furthermore, very similar heats of fusion were recorded for the samples with a known thermal history (ie cycle two); 86.09 Jg^{-1} for samples extracted using chloroform and 76.68 Jg^{-1} for samples extracted using DMSO. The differences observed in thermal cycles one and two suggest that different crystalline morphologies develop during the extraction and precipitation procedures, but once heated into the melt and cooled at the same rates, melt-crystallisation of both sample types yields very similar crystalline lamella thicknesses. The heats of fusion noted above (and also reported in table 1) suggests that samples extracted using DMSO are less crystalline, but this probably the result of differing

crystallisation kinetics which limit the development of crystallinity in the timescale imposed by the heating and cooling rates selected in the DSC experiment. Assuming no variations in the chemical structure, differences in the crystallisation kinetics may be attributed to variation in molecular weight.

Gel permeation chromatography (GPC) was used to determine the molecular weight of the P(3HB) samples extracted via both methods (Fig. 2c). Samples (250 mg) of each P(3HB) were dissolved in 50 mL chloroform at 85 °C for 2 hours under reflux, filtered using a 0.22 μm PVDF membrane and analysed using an Agilent 390-LC MDS instrument with differential refractive index (DRI) detector. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm) and a PLgel 5 μm guard column. The eluent was $CHCl_3$ with 2 vol. % triethylamine additive. Samples were run at 1 $mLmin^{-1}$ at 30 °C. Poly(methyl methacrylate) and poly(styrene) standards (Agilent EasyVials) were used for calibration. Experimental molar mass (M_n,SEC) and dispersity (Đ) values of synthesized polymers were determined by conventional calibration using Agilent GPC/SEC software.

The weight average (M_w) molecular weight of P(3HB) extracted with DMSO was 712 kDa whereas the M_w of chloroform / Soxhlet extracted P(3HB) was 604 kDa. The corresponding number average weights (M_n) were 473 kDa and 397 kDa respectively. It is apparent from Fig. 2c that the molecular weight distribution in the sample obtained from the Soxhlet extraction is reduced in comparison with the sample extracted using DMSO. This finding is in accordance with the observation that the crystallisation kinetics are reduced in the DMSO extracted sample; it is generally observed that the rate of crystallisation of a polymer is inversely proportional to molecular weight.

It is not clear why the extraction processes yield different molecular weight distributions, but the observation could be explained in terms of the effect of the development of additional

crystallinity (secondary crystallisation) in the samples as a result of storage at a temperature of 85 °C for a period of 8 hours. This time period would allow crystallisation in the sample to continue resulting in a progressive reduction in solubility that may exclude the dissolution of longer polymer chains resulting in what is in effect a fractionation process. However, this is clearly an area to explore in further work.

In summary, a rapid method of isolation of PHA from *C. necator* has been developed that eliminates the requirement for use of halogenated solvents. It should however be noted that this method of PHA extraction is not suited to large-scale extraction. Isolated P(3HB) samples purified using this method were thought to contain dimethylsulphide (DMS, identified by its characteristic cabbage-like aroma), probably generated by microbial reduction of DMSO entrained within the PHA. Further studies are needed to optimise the latter stages of the process in order to remove residual DMSO from the isolated PHA, eliminating formation of DMS. Nonetheless, this method offers a rapid, low-solvent approach to isolating small quantities of PHA for DSC and FT-IR analysis and as such has utility for screening experiments. Future work could investigate the effect of cellular PHA content on the ability of DMSO to lyse cells, and develop the technique for the isolation of PHA from other organisms.

Acknowledgements

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Figure Legends

Figure 1. Optimisation of DMSO lysis method using flow cytometry (FCM).

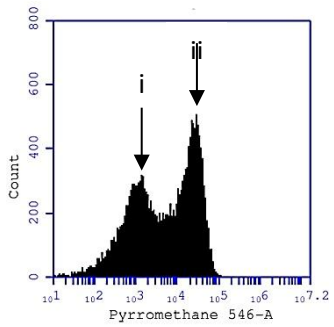
a) FCM analysis of *C. necator* DSM428 cells. X axis is Pyrromethene 546 fluorescence (488 nm laser excitation, 533/30 BP filter detection), signifying PHA content of individual bacteria; Y axis is number of bacteria. Population i, PHA^- bacteria; population ii, PHA^+ bacteria. b) OD_{600} of 50 mL of DMSO to which was added successive 250 µL aliquots of *C. necator* DSM428 cell suspension, each containing the equivalent of 18.7 mg dry biomass. The OD_{600} of DMSO and cells was far lower than expected; this was caused by DMSO-mediated lysis of bacteria. A linear relationship was observed until the equivalent of 380 mg dry biomass was added, after which the OD_{600} increased more rapidly upon addition of bacterial suspension. c) FCM analysis of the DMSO-bacterial suspension mixture at point 1 on panel (b). X axis is Pyrromethene 546 fluorescence, signifying PHA content of individual bacteria; Y axis is SYTO62 fluorescence (633 nm laser excitation, 670LP filter detection), differentiating cells (higher fluorescence) from non-cellular particles. One population is visible consisting of PHA^- bacteria. All the added PHA^+ bacteria had been lysed by the DMSO. d) FCM analysis of DMSO-bacterial suspension mixture at point 2 on panel (b). Two populations are visible: Population i is PHA^- bacteria which have not lysed, population ii is PHA^+ bacteria which are unable to be lysed as the DMSO has become saturated. e) As (d), but after 1 hours incubation, showing that the PHA containing bacteria (population ii) are still present. Data is representative of a number of repeated experiments.

Figure 2. a) FTIR spectra of P(3HB) extracted by DMSO extraction (solid trace) and comparative specta from P(3HB) extracted using the chloroform Soxhlet method (dashed trace) and commercial PHB (dotted trace). b) Differential scanning calorimetry traces for DMSO and Soxhlet extracted P(3HB). Both extraction methods display similar melting

points, however a 20 °C difference was observed in the crystallisation temperature. c) Molecular weight distribution of P(3HB) extracted using DMSO extraction (solid line) and the chloroform Soxhlet method (dashed line) determined using gel permeation chromatography.

Figure 1

A



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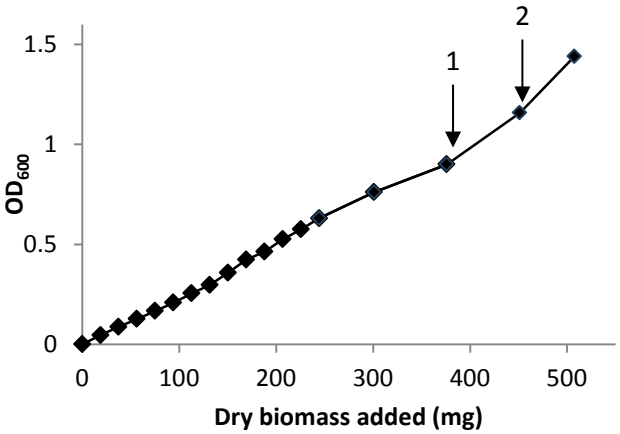

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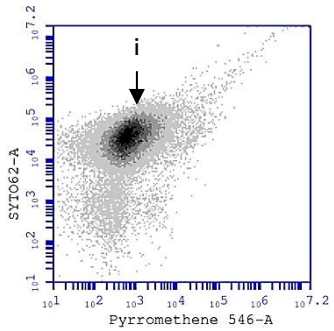

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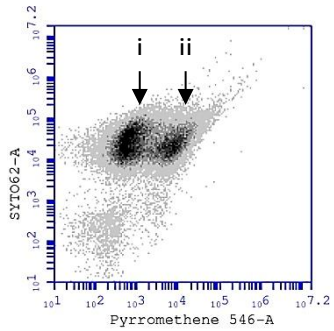

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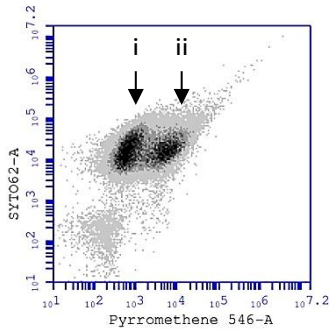
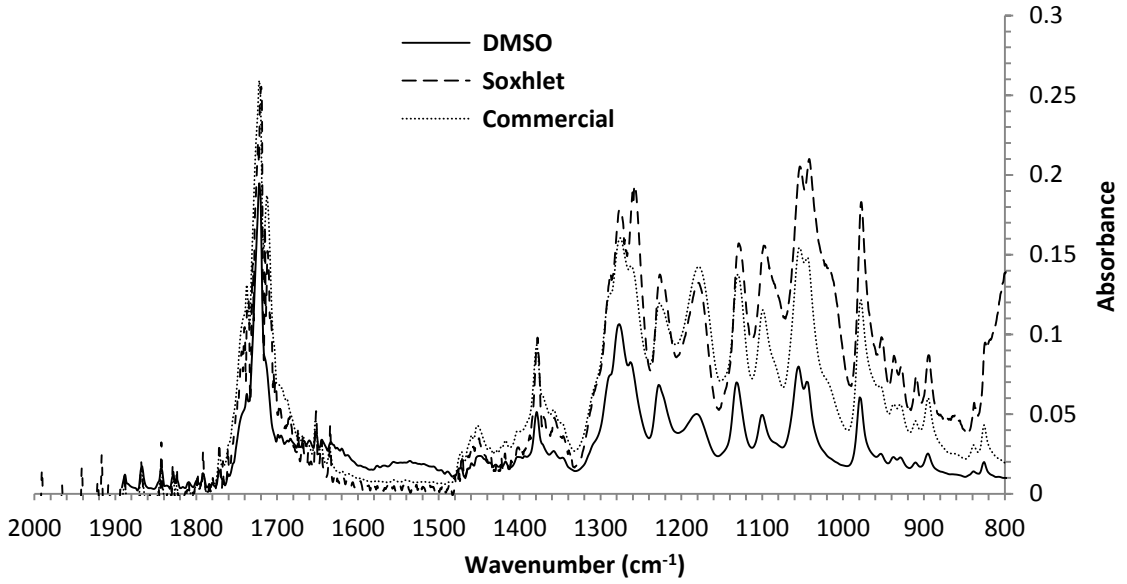


Figure 2

A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Soxhlet
Commercial
Absorbance
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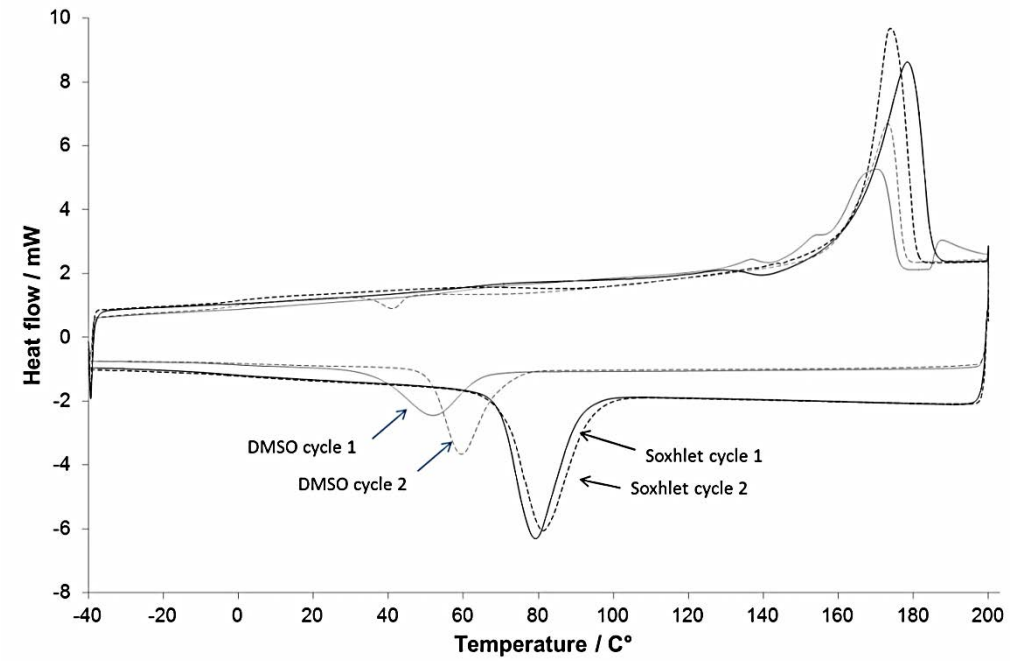
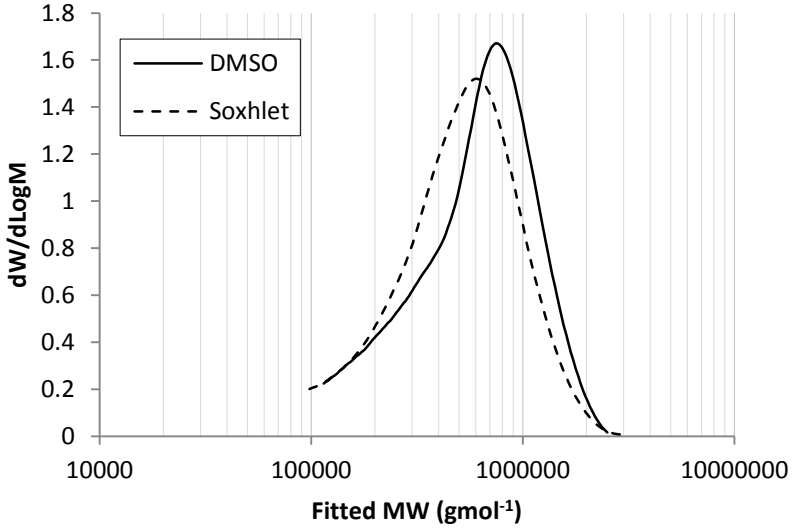
0.15
0.1
0.05
0
2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800
Wavenumber (cm-1)

B


Heat flow / mW
10
8
6
4
2
0
-2
-4
-6
-8
DMSO cycle 1
DMSO cycle 2
Soxhlet cycle 1
Soxhlet cycle 2
-40 -20 0 20 40 60 80 100 120 140 160 180 200
Temperature / C°

C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Soxhlet
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10000 100000 1000000 10000000
Fitted MW (gmol-1)

Table 1

Extraction method	Transition temperatures (°C)				Heats of formation (J g^{-1})			
	Cycle 1		Cycle 2		Cycle 1		Cycle 2	
	Heating	Cooling	Heating	Cooling	Heating	Cooling	Heating	Cooling
Chloroform	T_m 177.93	T_c 79.38	T_m 173.38	T_c 81.37	T_m 90.38	T_c -60.26	T_m 86.09	T_c -63.44
DMSO	T_m 169.92	T_c 51.90	T_c 40.98 T_m 172.86	T_c 59.61	T_m 79.15	T_c -38.89	T_c -3.70 T_m 76.68	T_c -48.87

Table 1. Summary of DSC data for P(3HB) extracted by chloroform and DMSO methods. The melting transitions are comparable between the two extraction methods, however the crystallisation temperature and heat of formation on cooling of the DMSO extracted sample are significantly lower.