

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

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

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11 **Key words:** Polyhydroxyalkanoate, Dimethyl Sulphoxide, Solvent extraction, Bioplastic.

12 **Abstract**

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

18

19 **Main text**

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

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

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

50 *C. necator* strain H16 (DSM428; DSMZ, Braunschweig, Germany) was grown in MSM  
51 medium using fructose as a carbon and energy source at a C:N ratio of 30:1 g/g, conditions  
52 under which poly-3-hydroxybutyrate is generated. MSM contained 2.3 gL<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 2.9 gL<sup>-1</sup>  
53 Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1 gL<sup>-1</sup> NH<sub>4</sub>Cl, 0.5 gL<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 gL<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.05 gL<sup>-1</sup>  
54 Fe(NH<sub>4</sub>) citrate and 5 mL trace element solution SL-6 (comprising 0.1 gL<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O,  
55 0.03 gL<sup>-1</sup> MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.3 gL<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 0.2 gL<sup>-1</sup> CoCl<sub>2</sub>·6 H<sub>2</sub>O, 0.01 gL<sup>-1</sup> CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.02  
56 gL<sup>-1</sup> NiCl<sub>2</sub>·6H<sub>2</sub>O and 0.03 gL<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O). Precultures were prepared in 250 mL  
57 conical flasks containing 20 mL of ME medium (5 gL<sup>-1</sup> Peptone and 3 gL<sup>-1</sup> Meat extract)  
58 inoculated with a loopful of *C. necator* and incubated for 24 h at 30 °C and 200 rpm. Two  
59 litre conical flasks containing 200 mL of MSM were inoculated with a volume of this  
60 preculture required to result in an optical density at 600 nm (OD<sub>600</sub>) of 0.1. Five millilitres of  
61 40 % (w/v) fructose solution was added to each culture after 24 and 48 hours. After 72 h  
62 growth, cultures were harvested by centrifugation and resuspended in phosphate buffered  
63 saline (PBS).

64 Addition of DMSO to *C. necator* H16 cell suspensions was found to rapidly clear the  
65 suspension, presumably by bacterial lysis. Conversely, DMSO did not lyse *C. necator* PHA<sup>-</sup>  
66 cells which cannot generate P(3HB). This suggested that DMSO enters the *C. necator* cells

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

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

79 *C. necator* H16 were resuspended in PBS at a concentration of 74.6 mg dry biomass  $\text{mL}^{-1}$ .  
80 Aliquots (250  $\mu\text{L}$ ) of this cell suspension were added to 50 mL of DMSO incubated at 70 °C  
81 with agitation. The  $\text{OD}_{600}$  of the DMSO and cell suspension mixture was measured after the  
82 addition of each aliquot of cell suspension (Fig. 1b). The measured  $\text{OD}_{600}$  of the DMSO and  
83 cell suspension mixture was far lower than expected, suggesting that the majority of cells  
84 lysed upon addition to DMSO. Further successive 250  $\mu\text{L}$  aliquots of cell suspension were  
85 added to the DMSO every 5 minutes. Flow cytometry was used to analyse the DMSO – cell  
86 suspension mixture during successive addition of cell suspension. Samples were doubly  
87 stained with Pyrromethane 546 and 0.4  $\mu\text{M}$  SYTO62 (Invitrogen; a DNA dye that stains all  
88 cells red). After addition of the equivalent of 380 mg of dry cells, flow cytometry revealed  
89 one population of cells that had a low concentration of PHA as determined by pyrromethene  
90 546 staining (Fig. 1c). This corresponds to *C. necator* cells that had not accumulated PHA; as

91 shown in Fig. 1a, a sub-population of cells fail to accumulate PHA in liquid culture.  
92 However, after addition of the equivalent of 450 mg dry cells, two populations were visible  
93 by flow cytometry (Fig. 1d): one comprising cells containing a low quantity of PHA; and one  
94 comprising cells containing more PHA, comparable to Fig. 1a. These populations were still  
95 present after an additional 1 hour of incubation at 70 °C (Fig. 1e). In addition, the gradient of  
96 the OD<sub>600</sub> versus biomass added graph increased after addition of the equivalent of 380 mg  
97 dry biomass, indicating that cells were no longer being effectively lysed by the DMSO (Fig.  
98 1b). Taken together, this indicates that 50 mL of DMSO could effectively lyse 380 mg of *C.*  
99 *necator* biomass containing P(3HB).

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

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

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

119 Yields from both methods were comparable: Soxhlet extraction yielded 2.51 mg of P(3HB)  
120 per mL of culture while DMSO extraction yielded 2.79 mg per mL of culture. FTIR was  
121 performed on solid pellets of P(3HB) which were placed directly under ATR (Thermo  
122 Nicolet 380 FTIR with Smart Orbit attached set to 'absorbance', a resolution of 1 cm<sup>-1</sup> and  
123 128 scans) for spectrum detection. In terms of peak locations, the FTIR spectrum of the  
124 DMSO extracted P(3HB) sample (Fig. 2a) was very similar to that of Soxhlet-extracted  
125 P(3HB) and commercial P(3HB) (Goodfellow Cambridge Ltd., Huntingdon, UK). General  
126 differences in peak intensities can be attributed to different clamping forces resulting from  
127 differing sample geometries and/or inhomogeneities. However, closer inspection of the  
128 spectra shows that a number of bands contain peaks in very similar locations, but with  
129 differing relative intensities. This may indicate differing crystalline morphologies (and degree  
130 of crystallinity).

131 P(3HB) extracted by DMSO and chloroform Soxhlet extraction were compared using  
132 differential scanning calorimetry using a Mettler Toledo DSC 1 (Mettler Toledo, Leicester,  
133 UK) calibrated with indium and tin standards. The P(3HB) samples (~5 mg) were weighed  
134 into a 40 µL aluminium DSC pan (Mettler Toledo) which was then capped with an  
135 aluminium DSC pan lid (Mettler Toledo) and sealed with a press (Mettler Toledo). The  
136 sample were subjected to the following programme, the temperature was held at -40 °C for 5  
137 minutes before raising it to 200 °C at a rate of 10 °C min<sup>-1</sup>. Following a 5 minute isotherm at  
138 200 °C, the sample was cooled to -40 at a rate of 10 °C min<sup>-1</sup>. This procedure was repeated  
139 twice in order to impose a known thermal history on the samples.

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

154 The thermal properties of P(3HB) extracted by the two methods are summarised in Table 1.  
155 On the first heating runs, the melting points of the two P(3HB) samples vary by 8 °C (DMSO  
156 170 °C and chloroform 178 °C) however, on cooling and reheating, the melting points are  
157 virtually identical at c.173 °C. Furthermore, very similar heats of fusion were recorded for the  
158 samples with a known thermal history (ie cycle two); 86.09 Jg<sup>-1</sup> for samples extracted using  
159 chloroform and 76.68 Jg<sup>-1</sup> for samples extracted using DMSO. The differences observed in  
160 thermal cycles one and two suggest that different crystalline morphologies develop during the  
161 extraction and precipitation procedures, but once heated into the melt and cooled at the same  
162 rates, melt-crystallisation of both sample types yields very similar crystalline lamella  
163 thicknesses. The heats of fusion noted above (and also reported in table 1) suggests that  
164 samples extracted using DMSO are less crystalline, but this probably the result of differing



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

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

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

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

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

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

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213

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230

231

232 **Figure Legends**

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

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

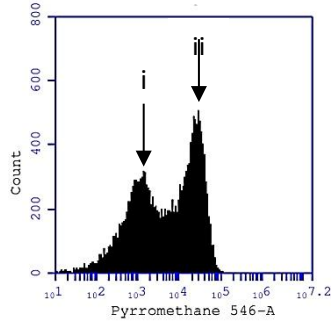
252 **Figure 2.** a) FTIR spectra of P(3HB) extracted by DMSO extraction (solid trace) and  
253 comparative spectra from P(3HB) extracted using the chloroform Soxhlet method (dashed  
254 trace) and commercial PHB (dotted trace). b) Differential scanning calorimetry traces for  
255 DMSO and Soxhlet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

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

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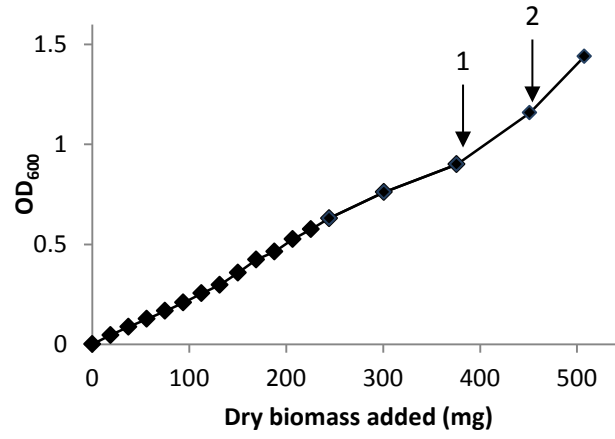
261 Figure 1

262 A

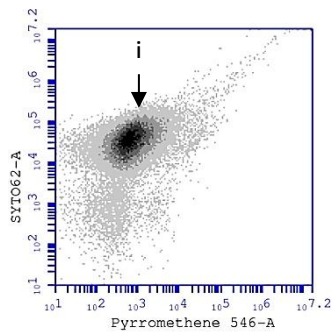


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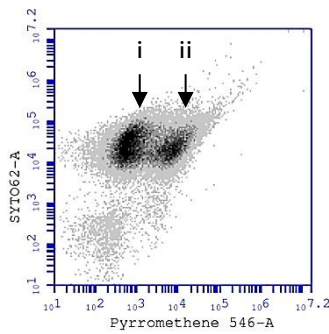
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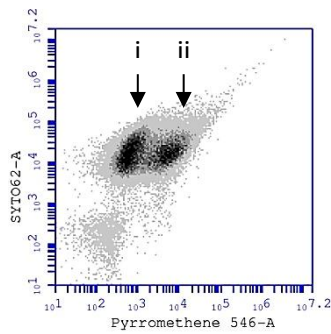
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D



E



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270 **Figure 2**

271 **A**

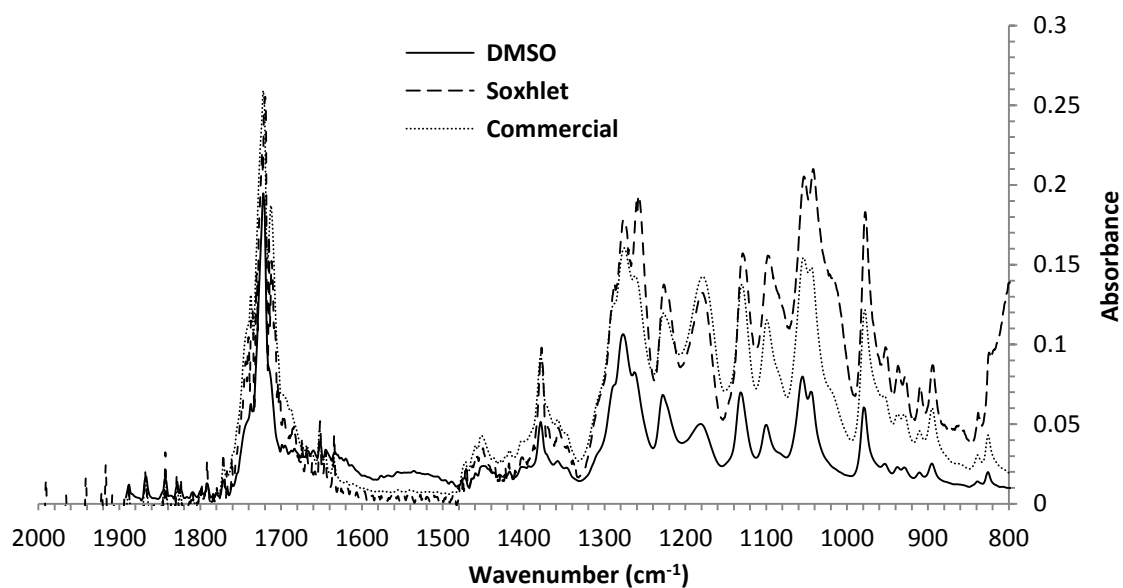
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277 **B**

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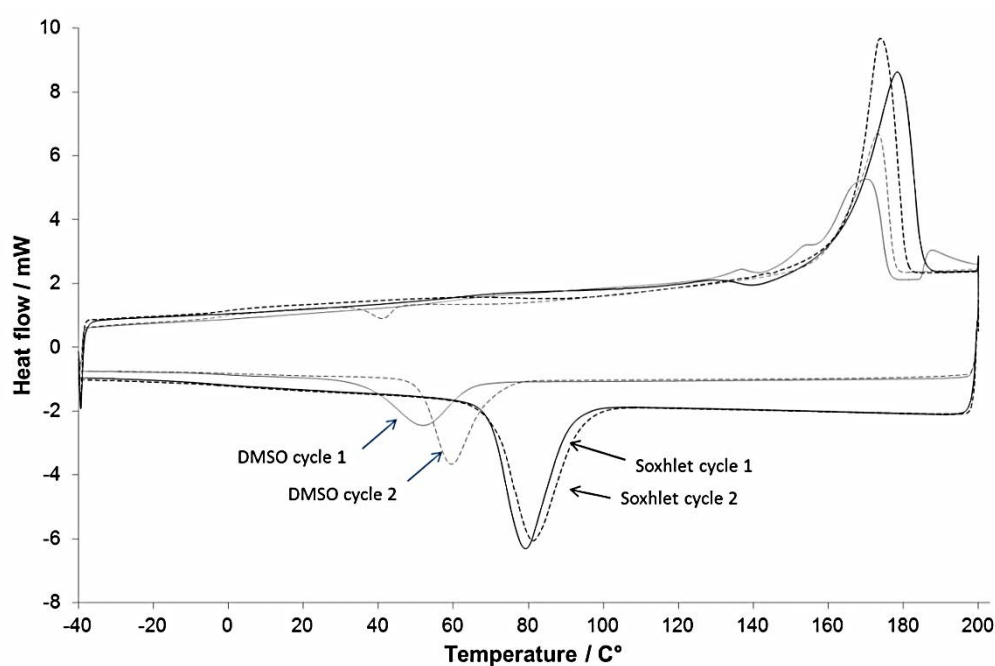
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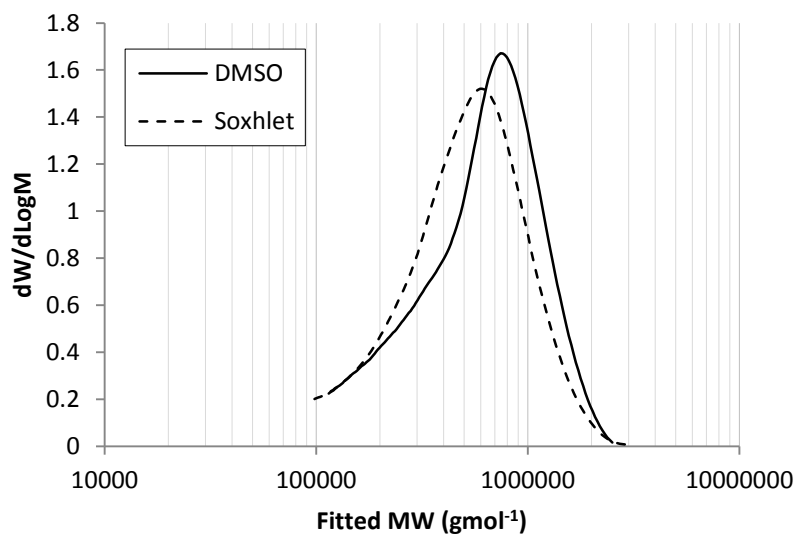
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285 **C**

286



287 **Table 1**

288

Extraction method	Transition temperatures (°C)				Heats of formation (J g <sup>-1</sup> )			
	Cycle 1		Cycle 2		Cycle 1		Cycle 2	
	Heating	Cooling	Heating	Cooling	Heating	Cooling	Heating	Cooling
<b>Chloroform</b>	T <sub>m</sub> 177.93	T <sub>c</sub> 79.38	T <sub>m</sub> 173.38	T <sub>c</sub> 81.37	T <sub>m</sub> 90.38	T <sub>c</sub> -60.26	T <sub>m</sub> 86.09	T <sub>c</sub> -63.44
<b>DMSO</b>	T <sub>m</sub> 169.92	T <sub>c</sub> 51.90	T <sub>m</sub> 172.86	T <sub>c</sub> 59.61	T <sub>m</sub> 79.15	T <sub>c</sub> -38.89	T <sub>m</sub> 76.68	T <sub>c</sub> -48.87

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

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