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TECHNICAL NOTE

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

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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 Fourier transform infrared spectroscopy and differential scanning calorimetry.

[**Key words:** Polyhydroxyalkanoate; Dimethyl sulphoxide; Solvent extraction; Bioplastic]

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 Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (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°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 h. After 72 h growth, cultures were harvested by centrifugation and resuspended in phosphate buffered saline (PBS).

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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, OH, 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 heterogeneity 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 min. Flow cytometry was used to analyse the DMSO—cell suspension mixture during successive addition of cell suspension. Samples were doubly stained with pyrromethene 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 h 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

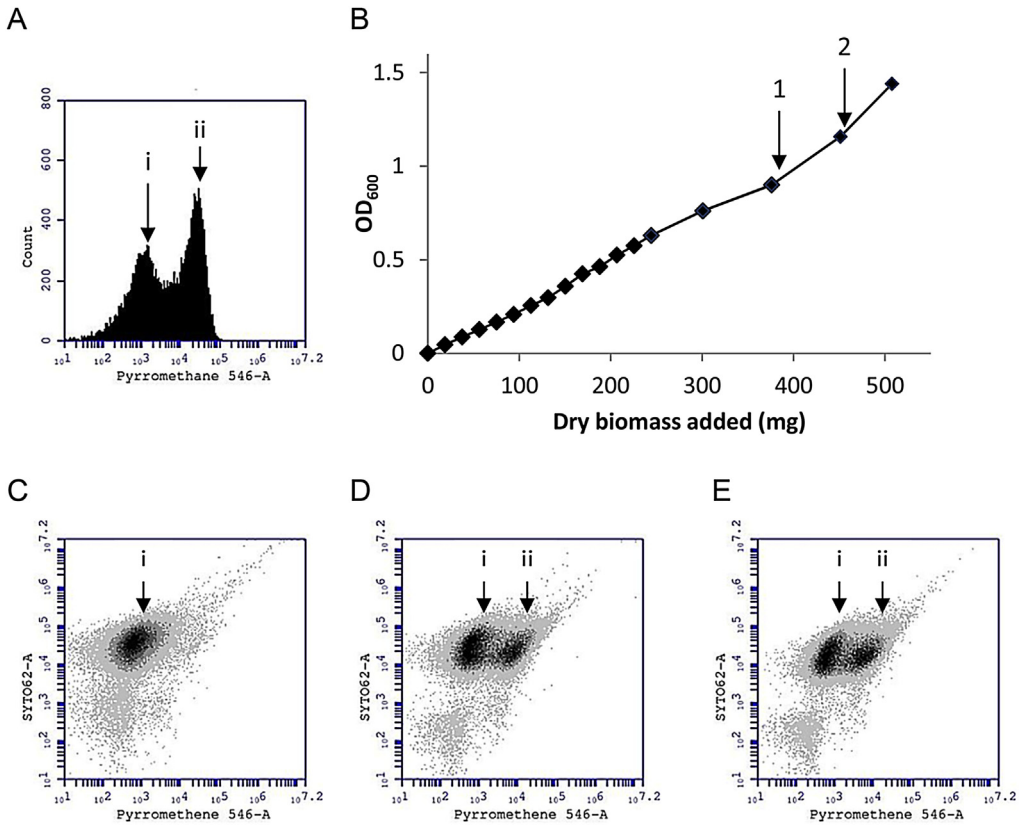

FIG. 1. Optimisation of DMSO lysis method using flow cytometry (FCM). (A) FCM analysis of *C. necator* DSM428 cells. X axis is pyrromethene 546 fluorescence (488 nm laser excitation, 533/30 BP filter detection), signifying PHA content of individual bacteria; Y axis is number of bacteria. Population i, 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 panel D, but after 1 h incubation, showing that the PHA containing bacteria (population ii) are still present. Data is representative of a number of repeated experiments.