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# Comparison of several methods for the separation of poly(3-hydroxybutyrate) from *Cupriavidus necator* H16 cultures

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ABSTRACT

Biopolymers, such as polyhydroxyalkanoates (PHA), are an environmentally friendly alternative to plastics derived from fossil fuels. However, producing PHA in a cost-effective way requires the development of highly efficient separation and purification treatments. In this study, one acid treatment (sulphuric acid combined with a subsequent bleaching step) and three alkaline treatments (sodium hypochlorite, sodium hydroxide and a combination of the latter with an halogenated solvent) were evaluated for recovering poly(3-hydroxybutyrate) (PHB), a type of PHA, from *Cupriavidus necator* H16 cells with high biopolymer content (65%). Purity, percent of recovery and the properties of the PHB obtained after each treatment, together with the costs and environmental impacts associated with each treatment, were determined and compared. The lowest recovery costs were obtained with the sodium hydroxide and sulphuric acid treatments (1.02 and 1.11 € $kg^{-1}$, respectively). Estimated $CO_2$ emissions of these two treatments were 18% of those based on the use of sodium hypochlorite. However, the highest purity (98%) and lowest polymer degradation were achieved with the acid treatment. Consequently, the acid treatment was selected as the most effective choice for PHA recovery.

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## 1. Introduction

A potentially interesting way to decrease the environmental impact of conventional petroleum-derived plastics is to replace them with biodegradable polymers. In this context, polymers of biological origin, such as polyhydroxyalkanoates (PHA) and among them polyhydroxybutyrate (PHB), play an important role. These polymers are accumulated inside bacterial cells as energy and carbon storage. *Cupriavidus necator*, the best-studied PHB-accumulating bacterium, stores PHB when there is an excess of carbon with respect to some other essential nutrient in the medium, such as nitrogen, phosphorous or oxygen [1,2]. To compete with conventional plastics, PHA production costs must be minimised. It has been estimated that more than 50% of the cost of PHB production are associated with the recovery and purification of the polymer [3]. Due to the large impact of the recovery step on production costs, efficient methods for separation and purification of PHA from PHA-containing cell mass are essential for producing bioplastics from renewable resources in a cost-effective and environmentally friendly way. The ideal method would maintain polymer properties and achieve high purity and recovery levels with low production costs. On an industrial scale, the use of halogenated solvents is the most common method for PHA extraction. However, this method has important drawbacks, such as its high chemical costs and its associated hazards [4]. Among the alternative recovery processes that have been proposed, digestion of the cells with chemicals or enzymatic cocktails and extraction with non-halogenated agents have received the greatest interest due to their simplicity and more affordable cost [5–8]. More recently, the use of chemicals that can selectively dissolve non-PHA biomass has also been proposed [9].

In this study, four separation processes that constitute promising alternatives to the commonly applied processes on an industrial scale were studied using a PHB-containing biomass. For each process, the processing conditions that achieved the highest purity and polymer recovery were determined. Then, the different treatments were compared considering each treatment's operational performance, economical and environmental criteria and final product characteristics.

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## 2. Materials and methods

### 2.1. PHB production

A mineral salt medium composed by 2.0 g L$^{-1}$ $(NH_4)_2HPO_4$, 2.1 g L$^{-1}$ $KH_2PO_4$, 0.2 g L$^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.1 g L$^{-1}$ $CaCl_2{\cdot}2H_2O$, 0.006 g L$^{-1}$ $FeCl_3{\cdot}6H_2O$, 0.1 mL L$^{-1}$ of trace element solution SL6 [10] and 30 g L$^{-1}$ of sodium gluconate was used to produce the *C. necator* H16 inoculum in a Biostat DL-30 reactor (with 25 L of medium).

Polymer accumulation with this strain was performed in a 400-L bioreactor (Biostat D650, Sartorius) for 68 h, using a fed-batch configuration into which gluconate was continuously fed to induce a nitrogen limitation [11]. A medium composed of 400 g L$^{-1}$ of gluconate, 5 g L$^{-1}$ $MgSO_4{\cdot}7H_2O$, 57 g L$^{-1}$ $(NH_4)_2HPO_4$ and 257 g L$^{-1}$ $NH_4Cl$ was fed during the first 40 h of operation at a rate that led to a constant gluconate concentration of approximately 17 g L$^{-1}$ in the reaction medium. Then, the cells were cultivated with an excess of carbon to attain nitrogen-limitation conditions.

### 2.2. PHB separation

*C. necator* H16 cells were separated by centrifugation and then freeze-dried. The particle size of the resulting solid was first homogenised by milling using a conventional blender (maximum speed, 30 s). Then, the homogenised solid was subjected to one of the following treatments to recover the polymer accumulated inside the cells. All experiments were performed in triplicate in closed 500 mL glass bottles with a volume of liquid of 100 mL, except for chloroform extraction.

#### 2.2.1. Chloroform extraction

Chloroform extraction is a well-established reference standard procedure. 1 mg mL$^{-1}$ of lyophilised cells was suspended in chloroform in closed glass tubes at 60 °C for 36 h [12]. Then, the polymer was precipitated by adding ten volumes of methanol and dried at 55 °C.

#### 2.2.2. NaOH treatment (PHB-R1)

Alkaline digestion of biomass was performed using a solid content of 2.5% w/v with NaOH solutions at different concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 N) for 4 h at 37 °C and 500 rpm. Then, the procedure was repeated with the optimal NaOH concentration selected in the previous step and various solids contents (2.5, 5, 7.5 and 10% w/v). Samples were centrifuged, and the solid phase was washed twice with water and once with ethanol and freeze-dried. In each washing step, approximately 0.8 mL of liquid per g of initial biomass was used.

#### 2.2.3. NaOCl treatment (PHB-R2)

Similarly, a commercial NaOCl solution (13% v/v) was used to perform biomass digestion at different solids contents (within the range 2.5–7.5%, w/v) at 37 °C and 500 rpm for 4 h. The solid was separated as described above.

#### 2.2.4. NaOCl and dichloromethane treatment (PHB-R3)

At the same temperature and agitation conditions and with a solids content of 2.5% (w/v), a combination of NaOCl solution (13% v/v) with a halogenated solvent, dichloromethane, in a 1:1 (v/v) ratio was used to digest the biomass and to extract the polymer. The polymer in the organic phase precipitated when 10 volumes of ethanol were added. The resulting solid was washed with water and ethanol and freeze-dried.

#### 2.2.5. Acid treatment (PHB-R4)

H2SO4 solutions at several acid concentrations were used to digest the biomass (5%, w/v) at different temperatures and for different times. Three levels were defined for each of these process conditions: (i) temperatures of 37, 65 and 100 °C, (ii) treatment times of 1, 15 and 30 h; and (iii) acid concentrations of 2.5, 5 and 10% (v/v). After the acid treatment, the pH value was set to 10 using a 0.5 N NaOH solution, and the solid was washed with water. Finally, a mild bleaching step with sodium hypochlorite at 3% was applied for 1 h to remove residual protein. The mathematical relationships of the response variables (purity and recovery percentages) to the independent variables (temperature, acid concentration and time) were represented by quadratic model equations. Finally, response surfaces for purity and recovery percentages were plotted using the software MATLAB$_{TM}$ Version 7.3.0 (The Mathworks, Inc).

### 2.3. Reuse of chemical solutions

After the biomass was chemically digested with alkali or acid and the resulting solution was centrifuged to recover the solids, different fractions of the liquid phase (20%, 40%, 60% and 80%) were mixed with fresh solution for reuse. This operation was repeated four times with each digestion agent.

### 2.4. Polymer quantification

The polymer percentage in the samples was determined using gas chromatography (GC). Samples were subjected to methanolysis in a solution with 1 mL of chloroform, 0.85 mL of methanol and 0.15 mL of $H_2SO_4$ [13]. The resulting esters were determined by gas chromatography in split injection mode with helium as the carrier gas (5 cm min$^{-1}$) using GC equipment with an FID detector and a PEG Permaphase column (60 m, 0.32 mm of diameter, 0.5 μm, Restek GmbH, Bad Soden, Germany) [14]. A commercial P(3HB) (Ref. 363502, Sigma-Aldrich) was used to determine the calibration curve.

Purity and recovery percentages of the polymer were calculated using Eqs. (1) and (2), respectively:

$$\text{Purity}\,(\%) = \frac{\text{polymer weight}}{\text{total sample weight}} \times 100 \quad (1)$$

$$\text{Recovery}\,(\%) = \frac{W_f \times P_f}{W_i \times P_i} \times 100 \quad (2)$$

where $W_i$ is the initial dry weight of the solid introduced into the recovery step (g); $W_f$ is the total dry weight of the solid recovered after the recovery step (g); $P_i$, $P_f$ represent the purity of the solid before and after the recovery process, respectively (%).

### 2.5. Polymer characterisation

#### 2.5.1. X-ray powder diffraction (XRD)

The crystalline structure of the samples was studied using an X-ray diffractometer (Philips X'Pert), which provides Cu *K*α radiation (40 kV, 40 mA), employing the powder method. Every scan was recorded in the range of 2θ = 2–50° in step-by-step mode with step size 0.02°.

#### 2.5.2. Differential scanning calorimetry (DSC)

In DSC, samples were heated from room temperature to 650 °C at a heating rate of 20 °C min$^{-1}$ in an $N_2$ atmosphere. The melting temperature and enthalpy of fusion ($\Delta Hf$) were calculated from the maximum and the area of the first endothermic peak, respectively, whereas decomposition temperature (*Td*) was calculated from the

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