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Research Article

Comparison of different solvents for extraction of polyhydroxybutyrate from *Cupriavidus necator*

Polyhydroxybutyrate (PHBs) have attracted much attention due to their biodegradability and biocompatibility properties. The main drawback to the commercial production of them is their high cost. The recovery of PHB from bacterial cytoplasm significantly increases total processing costs. Efficient, economical, and environment-friendly extraction of PHB from cells is required for its industrial production. In the present study, several nonhalogenated organic solvents (ethylene carbonate, dimethyl sulfoxide, dimethyl formamide, hexane, propanol, methanol, and acetic acid) were examined for their efficacy regarding recovery at different temperatures from culture broth containing *Cupriavidus necator* cells. The highest recovery percentage (98.6%) and product purity (up to 98%) were seen to be those of ethylene carbonate-assisted extraction at 150°C within 60 min of incubation time. Average molecular weight of the recovered PHB (1.3×10^6) was not significantly affected by the extraction solvent and conditions. The melting point of PHB extracted using ethylene carbonate was measured to be 176.2°C with an enthalpy of fusion of 16.8% and the corresponding degree of crystallinity of 59.2%. NMR and GC analyses confirmed that the extracted biopolymer was PHB. The presented strategy can help researchers to reduce the cost to obtain the final product.

Keywords: Extraction / Nontoxic / Organic nonhalogenated solvent / Polyhydroxybutyrate / Recovery

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1 Introduction

Polyhydroxybutyrate (PHB) is short-carbon-chain length polyester belonging to the bacterial polyhydroxyalkanoates (PHAs) which are formed as insoluble inclusion bodies in cytoplasm of a number of microbial species [1]. As of now, approximately 150 different hydroxyalkanoic acids are known to serve as constituents of these bacterial storage polyesters. PHB was among a family of PHAs that was first discovered, by Lemoigne in 1926, as a constituent of bacterium [2,3]. PHB is a good alternative to petrochemical-derived polymers and serves numerous applications in different industrial fields due to its thermoplastic property. This polyester is used because of its biodegradability, nontoxicity, biocompatibility, and its application in terms

of renewable resources. Because of some problems in terms of practical application of PHB, today, polymer blends are more widely accepted. It is cited that such blends will greatly widen the spectrum of possible applications by expanding the range of available physical properties [4,5].

Technological barriers to PHB recovery are the main concerns when it comes to microbial production of PHB on large scale where both of the recovery efficiency and purity of PHB are mainly dependent on the extraction method employed to isolate the polymer from bacterial cells. Many studies have reported different techniques for PHB recovery. For most part, these studies have had their focuses on developing an economic and efficient method of high yield and high purity wherein physical and chemical properties of the biopolymer are maintained. Solvent extraction is the most common method for the recovery of PHB [6,7]. Many researchers reported influences of different extraction solvents and techniques on the quality and properties of extracted PHB. Separation of PHB from the solvent is performed via solvent evaporation or precipitation in a nonsolvent. The most commonly used solvents for this purpose are chlorinated hydrocarbons such as chloroform that is, however, too toxic, expensive, and nonrecyclable. One way to minimize these

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Abbreviations: CDW, cell dry weight; DMFO, dimethyl formamide; DSC, differential scanning calorimetry; FID, flame ionization detector; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate

disadvantages is to replace the chloroform with a cheap and less toxic alternative in the course of PHB extraction [8–10].

The objective of the present work was to recover PHB from *C. necator* via solvent extraction using different solvents (i.e. acetic acid, hexane, propanol, methanol, DMSO, dimethyl formamide (DMFO), and ethylene carbonate). The work was based on a report by Terada and Marchessault [11] who reported capacities of different solvents for dissolving PHB. A series of solvents were chosen according to the report by Terada and Marchessault [11], so as to find whether these solvents can replace the chlorinated toxic chloroform or not. Our previous studies have shown that, temperature has significant effect on the recovery yield and the extracted PHB properties (i.e. thermal properties and molecular weight). In addition, heating time was found to be of slight effect on the PHB recovery yield [12, 13].

The approach described in this study is based on the method described for chloroform recovery systems (herein referred to as “standard method”) [13]. For each solvent, three different temperatures were applied for three trials with a contact time of 60 min for each solvent extraction trial, so as to investigate suitability of the solvent. To the best of our knowledge, this is the first study to compare these solvents, with their efficiencies assayed at different temperatures. Additionally, biomass pretreatment with hypochlorite sodium and its effect on efficiency and purity were investigated. Finally, the polymer obtained from the best experiment (as measured by the recovery yield) was characterized by differential scanning calorimetry (DSC) and average molecular weight determination.

2 Materials and methods

2.1 PHB production

C. necator ATCC 17699 was obtained from Persian Type Culture Collection. Before being used, all media were sterilized at 121°C in an autoclave. For inoculum preparation, the cells were grown in LB medium (containing bact tryptone, yeast extract, and NaCl) on a shaker at 200 rpm for 10 h at 30°C. The composition of the basal mineral salt medium used in this study was as follows: $(\text{NH}_4)_2\text{SO}_4$ at 2.0 g/L, KH_2PO_4 at 0.54 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 1.2 g/L, citric acid at 1.7 g/L, and trace metal solution at 10 mL/L. The trace element stock solution was composed of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at 2.25 mg/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at 10 mg/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at 2 mg/L, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 7\text{H}_2\text{O}$ at 0.23 mg/L, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ at 0.1 mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at 1 mg/L, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 0.6 at mg/L, and 35% HCl at 10 mL/L [14]. For the production media and inoculum development, fructose was used as the carbon source at a concentration of 20 g/L. Initial pH was adjusted to 7.0.

2.2 Extraction of PHB

For the extraction of PHB, the cells were harvested by centrifugation of 250 mL broth at 5000 rpm. The following procedures were then employed.

Chloroform extraction: The biopolymer was extracted from cells by stirring 1 g of cell pellet in 20 mL of chloroform for 48 h

at 37°C followed by a purification process by precipitation with one volume of ice-cold ethanol [15].

Solvent–hypochlorite dispersion extraction: Eight different organic solvents, namely chloroform, ethylene carbonate, acetic acid, DMSO, DMFO, propanol, methanol, and hexane were screened for PHB recovery from cell broth. In each case, cell broth was extracted with organic solvent in ratios of 1:1 v/v. The procedure was as follows: 1 g of wet biomass was treated with a dispersion containing 10 mL of 10% sodium hypochlorite solution in water, at 37°C for 1 h. The obtained mixture was then centrifuged at 4000 rpm for 10 min, with the pellet then suspended in 10 mL of solvent (chloroform or other solvents) before being transferred into a thermostatic water bath. The suspensions were stirred and heated at different temperatures (25, 50, and 100°C for propanol, methanol, hexane; 50, 100, and 150°C for DMSO, DMFO, and ethylene carbonate that possess high boiling points) with a cell pellet-to-solvent ratio targeting 0.1% w/v. Later, the suspension was centrifuged at 4000 rpm for 10 min. PHB was recovered from the solvent phase, and precipitated by one volume of ice-cold ethanol. The precipitated biopolymer was washed with distilled water and, finally, dried at room temperature. All the experiments were carried out in triplicate and averages of the obtained data were reported. For each case, standard deviation of the obtained data was calculated and reported along with the data, so as to ensure statistical reliability of the data [13].

2.3 PHB characterization

Cell dry weight (CDW) determination: The samples (10 mL of culture suspension) were centrifuged in predried and preweighted tubes at 10 000 rpm for 5 min. The pellet (wet cells) was resuspended in distilled water and centrifuged. The washed cells were dried in hot air at 90°C for a minimum time of 24 h after when CDW was measured [15].

Percent recovery yield calculation: The percentage of recovered PHB was calculated as average amount of PHB (g/L) after recovery divided by total amount of PHB in biomass (g/L) multiplied by 100. The total amount of PHB (g/L) was measured using GC analysis. Since the bacterial cells were cultured in large volumes before dividing the final biomass into equal parts, the total crude amounts of PHB measured using GC analysis were similar in all experiments (≈ 0.3 – 0.4 g/L).

^1H NMR spectroscopy: The PHB extracted using ethylene carbonate and DMSO was suspended in DMSO- d_6 (deuterated DMSO) (10 mg/mL) to determine the structure of PHB. Such a low concentration necessitated the use of a higher number of scans. ^1H NMR spectra were recorded on a Bruker Avance spectrometer at 400 MHz (^1H) at 25°C. Tetra methyl saline was used as external reference and chemical shifts were reported in ppm [16].

DSC: Melting point (T_m), degree of crystallinity (X_C), and enthalpy of fusion of the samples (ΔH_f) were determined using a differential scanning calorimeter (DSC-822 calorimeter Mettler Toledo, Columbus, OH). At least 5 mg of each sample was used for each run that was conducted within the range of -25 to $190^\circ\text{C}/\text{min}$ at a heating rate of $10^\circ\text{C}/\text{min}$. The analyses were performed under a flow of N_2 at 20 mL/min [17].

PHB average molecular weight: High molecular weight of the biopolymer solution contributed to very high viscosity of the biopolymer solution compared to that of pure solvent. The recovered PHB samples via different organic solvents were analyzed by a viscometer (Ubbelohde dilution viscometer), so as to calculate viscosity against average molecular weight. In this method, viscosity of recovered PHB dissolved in chloroform is measured by comparing the flow times of two liquids of equal volumes (at room temperature) using the same viscometer. The concentration of PHB in solutions was adjusted to have a relative viscosity ranging within 1.1 to 1.8 [18]. From the Mark–Houwink equation, the relationship between molecular weight and viscosity is as follows:

$$[\eta] = 0.0119 \times 10^{-4} M_v^{0.78},$$

where $[\eta]$ is intrinsic viscosity of PHB in chloroform and M_v is molecular weight.

GC analysis: Composition and yield of PHB in cell pellets were determined by GC. Dried cells were mixed with sulfuric acid/methanol (1:5 v/v) and chloroform (1:1 v/v) and heated to 100 °C for 140 min to form methyl ester monomers. 0.5 mL of the methyl ester samples were injected into a GC equipped with a flame ionization detector (FID) and a Chrompack SE-30 capillary column. The injection part was held at 280°C. The oven temperature was kept constant at 100°C for 3 min before being increased at 10°C/min to separate the methyl esters. The FID detector temperature was increased to 300°C. PHB calibrations were performed with poly 3-hydroxybutyric acid standard (Sigma-Aldrich Chem) and benzoic acid (Sigma, USA).

3 Results and discussion

3.1 Choosing appropriate standard method

At first, efficiency of chloroform extraction with or without hypochlorite pretreatment was evaluated for choosing a standard chloroform method. Two different techniques, namely chloroform extraction and chloroform–hypochlorite dispersion extraction, were tested in this step. The chloroform–hypochlorite dispersion extraction involves polymer extraction from pretreated cells by hypochlorite using boiling chloroform. The resulting solution is then precipitated using one volume of ethanol, leaving residual lipids in solution. In this technique, the anionic hypochlorite is used to remove cellular debris before the intracellular polymer is dissolved in chloroform. On the other hand, the chloroform extraction method relies solely on solubility of the PHB in chloroform and does not involve any treatment with sodium hypochlorite. This method is also widely used to recover PHB [19].

In this work, the hypochlorite–chloroform dispersion method gave higher yield of the polymer (96%), followed by the chloroform method (82%). The level of PHB purity was then calculated from the total amount of PHB in the isolated powder as determined by comparing GC results to the crude weight of polymer powder. The results were verified by comparing them with those of standard PHB obtained from Sigma–Aldrich. It was found that, the purity of PHB extracted with dispersion

extraction method (99%) was higher than that by the chloroform extraction method (85%). This could be attributed to the increase in cell permeability with hypochlorite and the removal of cell debris and residual lipid from slurry in the hypochlorite dispersion extraction method.

Since, in preliminary results, the recovery yield and purity of recovered PHB were higher with chloroform–hypochlorite extraction system, pretreatment with hypochlorite sodium was done for the rest of experiments using different organic solvents. Additionally, chloroform–hypochlorite extraction was used as the reference method to which other approaches based on alternative organic solvents were compared.

Hypochlorite extraction is known to cause degradation of PHB. However, the extent of this degradation varies considerably among different organisms. In the experiments considered herein, the amount of PHB degraded to a compound of lower molecular weight during the recovery process was not significant (from average molecular weight analysis). The polymer extracted with hypochlorite–chloroform had a molecular weight of 1.5×10^6 . The molecular weight of the PHB was in the range of the values reported in the literature ($1.0 \times 10^4 - 4.0 \times 10^6$) [20, 21].

Thermal properties of PHB including glass transition temperature (T_g) and melting point (T_m) obtained by chloroform–hypochlorite extraction were 4°C and 175°C, respectively. Similar values were reported for chloroform–extracted PHB in literature (i.e. glass transition temperatures varying in the range of –5 to 5°C and melting temperatures ranging within 174–180°C). The obtained degree of crystallinity indicates that the corresponding PHB is a rigid and brittle material; the characteristics may limit its use in some final applications requiring high impact resistance [22].

3.2 Effects of extraction solvent and temperature on PHB recovery yield

Currently, several procedures are published for the extraction of PHB from biomass of different organisms. Although such approaches are known to provide promising routes, there still remain obstacles to efficient and cost-effective PHB recovery at desired levels of quality and purity. Additionally, many of the efforts have had their focuses on recovery using halogenated hydrocarbon solvents. Indeed, the use of nonhalogenated solvents will reduce the risk of pollution for the operators and for the environment [6, 23]. In this study, a process was designed for the recovery of PHB from bacterial wet biomass. Various organic solvents (ethylene carbonate, methanol, propanol, acetic acid, hexane, DMSO, and DMFO) were used for the extraction of PHB based on the reports by Terada and Marchessault [11] who theoretically evaluated PHB solubility of different solvents for industrial scale-up processes.

During the course of the present research, the solvents were tested for their ability to extract PHB at high yield and quality. The tests were conducted in a range of temperatures and after treatment with sodium hypochlorite. The temperature was taken as a design variable because of its significant effects on solvent extraction, according to our previous reports [12]. Additionally, physical properties of a solvent determine how effective

Table 1. PHB recovery yield (%) and purity (%) recovered by different solvents from wet biomass (the digits greater or less than 5 were rounded in all experiments)

Solvent	Extraction temperature (°C)	Recovered PHB (average) (g/L)	Recovery yield (%)	Purity (%)
Ethylene carbonate	50	1.14	82.6 ± 1	97 ± 1
	100	1.2	90.1 ± 1	99 ± 2
	150	1.32	98.6 ± 2	98 ± 1
Methanol	25	0.12	66.71 ± 1	98 ± 1
	50	0.14	81.16 ± 1	99 ± 2
	100	0.124	72.6 ± 2	97 ± 1
Propanol	25	0.072	16.02 ± 1	98 ± 1
	50	0.104	23.15 ± 1	99 ± 2
	100	0.128	28.49 ± 2	97 ± 1
Acetic acid	25	0.064	14.24 ± 2	98 ± 1
	50	0.068	15.13 ± 1	99 ± 2
	100	0.12	36.71 ± 2	97 ± 1
DMSO	50	0.064	20.6 ± 1	97 ± 1
	100	0.082	40.1 ± 1	99 ± 2
	150	0.16	60.6 ± 2	95 ± 1
DMFO	50	0.028	10.16 ± 1	85 ± 1
	100	0.056	20.5 ± 1	93 ± 2
	150	0.076	30.1 ± 2	97 ± 1
Hexane	25	0.056	2.3 ± 1	82 ± 1
	50	0.064	2.6 ± 1	89 ± 2
	100	0.068	2.8 ± 2	83 ± 1
Standard	100	0.4314	96	98 ± 1

All experiments were done in duplicate.

the solvent will be in an industrial recovery process. Except for DMSO, all alternative solvents were safe and had low toxicity. For most part (except for DMSO and ethylene carbonate), the solvents' densities were lower than 1 (water density), permitting easy removal of the PHB solution after the extraction (such low density solvent would separate and stay on top of water rather than mixing with cell debris).

In order to screen solvents' efficiencies for PHB recovery, PHB was extracted from wet cells containing 81% PHA of CDW according to materials and methods. Chloroform was used as a control solvent and succeeded to recover almost all PHA content of the cells (98%) at 100°C.

The content of extracted PHB varied in response to different organic solvents and extraction temperatures. Yield of extracted PHB varied from 2.3 to 98.6%, while the purities of all recovered polymer samples were higher than 95% (Table 1). The recovery yield increased gently with increasing temperature, while purity of extracted PHB was not significantly influenced by the temperature (Table 2). At lower temperatures, extraction was insufficient, as evidenced by the low yields obtained. However, the increase in temperature increased the disruption of cells and the solubility of PHB in the solvents. Extraction with ethylene carbonate had the highest yield of 98.6% at 150°C and 90.1% at 100°C, followed by DMSO (81.16 at 50°C). The lowest extraction yield ($\approx 2\%$) was that of hexane with which an increase in temperature had no significant effect on recovery yield. In most of the extracts, recovery yield increased strongly with increasing

Table 2. PHB solubility in different solvents are listed (the solvents are arranged according to their PHB solubility)

This study	Tereda et al. (Theoretically)	Kurdikar et al.
Ethylene carbonate at 150°C	Ethylene carbonate	Ethylene carbonate at 120°C
DMSO at 150°C	Acetic acid	DMSO at 117°C
Propanol at 100°C	DMSO	Acetic acid at 116°C
DMFO at 150°C	Propanol	DMFO at 90°C
Acetic acid at 100°C	DMFO	–
Methanol at 100°C	Hexane	–
Hexane at 100°C	Methanol	–

temperature, except for methanol for which the highest recovery yield was achieved at a temperature near its boiling point. It can be said that, almost all solvents (except for hexane) were capable of effectively dissolving this polymer. Here, the recovery from wet cells eliminated a biomass drying step from the downstream process, saving time, and cost [23, 24].

Lafferty and Heinze [25] demonstrated PHB recovery from moist cells on an industrial scale using cyclic carbonates like ethylene carbonate. In agreement with the present study, they showed that ethylene carbonate is a good solvent for dissolving PHB during the extraction. Generally, ethylene carbonate has been regarded as a safe solvent system for extraction of medicinally important metabolites or cosmetics.

In the aforementioned study, 25 mL of ethylene carbonate was added to 4 g of wet cells with a PHB content of 2.7 g. The mixture was heated up to 110°C while being stirred for 3 h. Polymer was then precipitated by cooling the mixture down to about 40°C and having the cooled mixture washed with water. A recovery yield of 92% was obtained, with no data reported on purity of the product obtained by these extractions. In the present study, the recovery yield obtained by the ethylene carbonate extraction using wet cells as starting materials on a 250 mL scale was found to be 98.6%, i.e. much higher than the 92% recovery yield observed by Lafferty and Heinze. After extraction, these cyclic carbonates can be reused with no need for regeneration steps. In addition, they can be used as solvents for PHB processing (e.g. in wet spinning). It is proved that the use of the cyclic carbonates imposes no significant effect on depolymerization of PHB even at high temperatures ($\approx 150^\circ\text{C}$). The residual cell mass from the ethylene carbonate extraction using wet cells showed very low PHB concentration, and the concentration of PHB in ethylene carbonate mixture was by far higher than other solvents. Additionally, since ethylene carbonate/PHB mixture is not as viscous as chloroform mixture, it can be filtered on industrial scale.

From Table 2 it can be concluded that, for a recovery process wherein wet cells are used as starting material, the solvent/precipitant pair of ethylene carbonate/ethanol represents a good alternative to standard chloroform method. Relatively high boiling point of ethylene carbonate (240°C) prevents it from being evaporated to environment at ambient temperatures and makes it reusable for several cycles of purification. This can cut the solvent consumption—an economic advantage. Besides, ethylene carbonate is considered safe due to its low toxicity.

Table 3. Thermal properties of PHB samples extracted with different solvents

Sample	X _c (%)	T _m (°C)	ΔH _f (J/g)	T _g (°C)	T _c (°C)
Commercial PHB	76.1	173.6	75.67	3.8	42
PHB extracted by ethylene carbonate	59.2	176.2	16.8	4.8	41
PHB extracted by DMSO	57.3	176	17.04	5.1	35.8

Kurdikar et al. [26] also studied PHB solubility of different organic solvents (such as ethylene carbonate). Even though the yield of recovery by the proposed method in the present research was comparable to that reported by Kurdikar et al. [26] the applied temperature in the proposed method was much lower than their studies, further increasing the safety of the process.

Table 3 presents the solvents as ranked based on their ability for dissolving PHB from three different literatures. According to Kurdikar et al., acetic acid, DMFO, DMSO, and ethylene carbonate are good PHA solvents. Being in agreement with Kurdikar et al., the present work showed that ethylene carbonate, DMSO, DMFO, and acetic acid are good PHA solvents.

Propanol, methanol, and hexane are known as poor PHA solvents at room temperature or temperatures below the corresponding solvent's boiling point, according to Terada and Marchessault and Kurdikar et al. [11, 26]. However, the experiments in the present research proved that, capabilities of these poor solvents can be enhanced with increasing temperature (except for hexane), so as to have the solvents improved in terms of their ability for dissolving PHB. For example, propanol is known as a poor PHA solvent in many literatures; however, with increasing the temperature it can compete with many good PHA solvents. This may be because of thermoplastic behavior of PHB (for most part, thermoplastic polymers can be dissolved in a wide range of solvents by adjusting the temperature, as opposed to thermosetting resins that are soluble in a few solvents only).

To recover the dissolved polymer from solvent/PHB mixture, we chose to precipitate the biopolymer with ethanol rather than evaporating or cooling the solvent. Evaporation can be problematic in batch operations where the polymer tends to coat the vessel after the solvent is removed. Additionally, any contaminants in the solvent (such as residual lipids) will copurify with the PHB. Upon precipitation with one volume of ice-cold ethanol, PHB-enriched solvent mixture typically gives rise to the formation of a precipitate that can be easily removed from the solvent. In contrast, in some systems used for PHA extraction, subsequent recovery of PHA is performed by reducing the temperature, generally resulting in PHA-enriched solvents that form a stable gel upon cooling [23]. Additional steps are then required to separate PHA polymer from the gel. In the present research, it was determined that by adding one volume of the precipitant to PHB/solvent solution, the polymer was precipitated sufficiently. Using only one volume of ethanol as PHA nonsolvent in this approach, one can overcome the problem of 10 volumes of PHA nonsolvent consumption for the precipitation of the PHB in other reports [27].

The method used to extract PHB (especially with ethylene carbonate) in the present study is simple yet efficient, yields highly purified PHB, and is economically much more

feasible than previously reported methods. One may reduce extraction costs using this method provided this method can be developed and extended for the extraction of various types of PHAs.

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3.3 Molecular weight determination

Molecular weight of a biopolymer is an important factor when it comes to its industrial usability. For most applications, it is desirable to have PHB with a molecular weight greater than 5×10^4 Da. A suitable solvent and process for the recovery of PHB is the one that causes no significant alteration in molecular weight of the biopolymer [28].

Viscosity of the biopolymer solution is a direct measure of hydrodynamic volume of the biopolymer molecules that is governed by the molecular size or the chain length and hence the molecular weight. The molecular weight of PHB extracted using chloroform (standard) was 1.5×10^6 . This is comparable to the molecular weight reported by Sim et al. [29] (i.e. $1.0 \times 10^4 - 4.0 \times 10^6$). Usually, a PHB of a molecular weight of 6×10^5 Da is acceptable for manufacturing thermoplastics.

Intrinsic viscosity and calculated average molecular weight of PHB extracted with different solvents at 100°C were approximately 14 dL/g and 1.4×10^6 , respectively. It can be said that, molecular weights of PHB samples obtained using different solvents were approximately the same, showing that the method and temperature settings used in this study did not cause severe degradation of the biopolymer. However, according to previous studies, it is better to set a limit to the temperature as the solvent begins to degrade the biopolymer at too high temperatures (particularly temperatures well-higher than the boiling point of the solvent). In this study, setting the temperature to 100°C, no significant difference was observed in molecular weight as compared to that of chloroform-extracted PHB.

3.4 Thermal properties

This section is focused on the PHB extracted by ethylene carbonate and DMSO, acknowledging their higher yields of recovery

Figure 1. DSC thermograms of (A) commercial PHB (B) biopolymer extracted by ethylene carbonate (C) biopolymer extracted by DMSO.

from wet cells, as compared to other solvents. Accordingly, thermal behavior of the PHB samples extracted by these two solvents was analyzed by a DSC (Fig. 1). The DSC curve showed an endothermal base-line shift associated with the T_g followed by a cold-crystallization exotherm (T_c) and, finally, a T_m . Appearance of the T_g and T_c in the curve confirmed the amorphous state of the biopolymer. The crystallinity values obtained for the extracted PHB samples using both solvents were found to be lower than that of commercial PHB, indicating the dependency of the crystallinity of the extracted PHB on the employed solvent.

Table 3 shows thermal properties (i.e. degree of crystallinity (X_c), T_m , T_g , and enthalpy of fusion (ΔH_f)) of the extracted biopolymers and standard samples. The reported data on this table shows almost the same melting points for the extracted PHB samples by these two solvents and commercial PHB. This feature is also comparable to those determined by others [22,30]. Therefore, the T_m of PHB was not significantly affected by the use of either ethylene carbonate or DMSO solvent at high temperatures.

The ΔH_f of the extracted PHB samples by ethylene carbonate and DMSO decreased from 75.76 J/g to 16.8 J/g and 17.04 J/g, respectively, potentially showing more elastomeric nature of extracted biopolymer with nonchlorinated solvent and resulting in an improvement in the processability and elongation of the biopolymer [30].

3.5 NMR

NMR spectroscopy has been proven to be a powerful tool for the determination of the structure of polymers. ^1H NMR was performed to verify the structure of the isolated biopolymer from *C. necator*. The ^1H NMR spectra of the PHB obtained from ethylene carbonate and DMSO extractions (Fig. 2) indicated the presence of pure PHB in all cases. The figure shows a number of characteristic PHB peaks at 5.2, 1.3, and 1.2 which correspond to a methine ($-\text{CH}$), methylene ($-\text{CH}_2$) multiplet, and methyl ($-\text{CH}_3$), respectively. Moreover, two relatively small peaks observed at 0.9 and 1.6 may be due to impurities. As an evidence of this finding, one can refer to the work by Rohini et al. [31]. The peaks at 2.5 and 3.3 are attributed to DMSO- d_6 and HOD, respectively.

4 Concluding remarks

In this work, feasibility of purifying PHB by seven nonhalogenated solvents from *C. necator* was studied. Among the considered solvents, ethylene carbonate, as an environment-friendly and recyclable compound, gave the best results (a recovery yield and purity of 98.6 and 98%, respectively) when the extraction was performed from wet biomass at 150°C. Additionally, regarding its low toxicity and high boiling point, ethylene carbonate can be reused several times without further purification. The purity and recovery yield obtained were comparable to those reported for chloroform extraction methods. Limited studies have been conducted on the use of biodegradable and biorenewable solvents for PHB extraction; the present study provides further

Figure 2. ^1H -NMR spectra of biopolymer extracted by (A) ethylene carbonate (B) DMSO. Methyl, methylene, and methine groups are indicated.

data for such a possibility. Recovery yield of hexane was too low even at high temperatures, indicating the capability of this organic solvent to serve as a very good nonsolvent (or precipitant) for PHB extraction, as an alternative to other precipitants.

The proposed recovery and purification method largely influenced the quality of the product for thermoplastic applications. The purified PHBs by ethylene carbonate and DMSO exhibited thermal stability and molecular weight comparable to those of commercial PHB. GC and NMR analyses clearly confirmed that the obtained polymer was PHB.

Totally, it can be said that solvent cost accounts for 20% of total PHB production cost. The PHB cost is about three euro per kilogram. Considering solvent cost as 20% of the total PHB

production cost. It stands approximately about 0.6 euro of total price. The price of ethylene carbonate is considered to be 1/2 of the chloroform price. It can be said that by using ethylene carbonate the industry can save almost 10% of the total PHB cost.

Practical Application

The feasibility of purifying PHB by several solvents by sodium hypochlorite pretreatment was investigated. The purity and recovery yield obtained were comparable to those reported for standard methods. This study shows that biomass pretreatment with hypochlorite sodium improve the efficiency and purity. The chosen solvents were cost-effective, environmentally friendly, and recyclable. Other PHB recovery approaches require the use of large volumes of the precipitation solvents to precipitate biopolymer from a PHB-enriched solvent. The recovery and purification method had a large influence on the quality of the product for thermoplastic applications. PHB purified by ethylene and DMSO displayed thermal stability and molecular weight comparable to commercial PHB.

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