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Evaluation and Characterization of Polyhydroxyalkanoates (PHAs) from Renewable Resources

Joyee Saha[1], Titly Ahmed[2], Lina Rafi Taposhi[3] and Dr. Md Salatul Islam Mozumder[4]
[1,2,3,4]Department of Chemical Engineering and Polymer Science, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh.
E-mail: [1]joey02saha@gmail.com, [2]nafisaammin007@gmail.com, [3]linataposhi02@gmail.com, [4]salatul-cep@sust.edu

Abstract

Plastic pollution has become an alarming issue in recent times due to its harmful impacts on human health and ecosystems. A promising solution to plastic pollution can be switching to biodegradable polymers like Polyhydroxyalkanoates (PHAs), which are greener alternatives of fossil resources-based plastics. This study has been conducted to optimize the conditions for the maximum production of PHA using pure culture bacteria (Cupriavidus Necator DSM 545) and hydrolyzed sucrose as a potential low-cost substrate. PHA was produced by a temperature and pH-controlled fermentation process in nutrient-rich growth media. The resulting dried biomass yielded a maximum PHA content of 70%. The investigation further compared two polymer extraction methods—solvent-based (using chloroform) and bio-based (using Bacillus Pumillus FH9). The thermal properties and the % purity of polymer were studied by TGA and DSC analysis. Maximum polymer purity of 87.87% and 98.1% were obtained using bio-based and solvent-based extraction respectively.

Keywords: Polyhydroxyalkanoates, Biodegradable polymer, Renewable, PHA Extraction.

1. Introduction

PHAs or Polyhydroxyalkanoates are a class of biodegradable thermoplastic polyesters that are produced by certain microorganisms under specific growth conditions. These biopolymers exhibit high biodegradability, biocompatibility and sustainability [1]. It has been found in studies that PHAs can be fully degraded within 28 days in seawater or freshwater conditions which makes them environment-friendly, sustainable replacement of petroleum-based plastic. [2].

Merely 10% of the plastic produced globally is recycled, with the majority being disposed of in land-fills where it releases harmful compounds that contaminate the soil and groundwater [3]. However, over the next 5 years, the capacity of the world's bioplastics production is predicted to more than triple, from approximately 2.4 million tons in 2021 to 7.5 million tons in 2026 [4] which makes renewable resources-based biopolymers like starch derivatives, polylactic acid, cellulosic polymers and Polyhydroxyalkanoates lucrative alternatives for study.

RO-water or distilled water is expensive. Since a huge volume of growth media needs to be prepared with sufficient nutrients for the growth of the bacteria, the cost of water supply can contribute a large sum to the overall cost of production of polymer. In order to determine how the water costs can be maximally reduced, the effect of different water sources on the production process of PHA from bacteria was studied extensively, by using RO-water, regular tap-water (from SUST), commercial drinking water (“Fresh” brand), and mineral media enriched with TES (trace element solution). Another major cost source is the substrate. Using cheaply available carbon sources as substrates, that may also include wastes, is one of the several ways of cost reduction. Based on [5], monosaccharides (like glucose and fructose), as well as disaccharides (like sucrose and lactose), have been known to accumulate PHA. Especially, glucose and fructose have been known to yield Polyhydroxybutyrates (PHB) [6]—which is the 4-carbon member of the family of PHAs.

The current study focused on assessing the possible viability of using thick liquor waste from the sugar industry by using concentrated hydrolyzed sucrose in lab-scale. The scope of cost reduction using different water sources was also analyzed. Moreover, the effect of different initial nitrogen concentration on PHB

production was also explored. This study also developed a comparison between a bio-based and a solvent-based extraction method. Finally, whether the type of extraction process f had any impact on the thermal properties of the polymer was also evaluated.

2. Materials and methodology

2.1. Materials

All chemicals used were procured from PT SMARTLAB (Indonesia); only $MgSO_4$ was from RESEARCH LAB FINE CHEM INDUSTRIES (India). The glassware used were made out of Pyrex. Organism used as PHA-producing bacteria was *Cupriavidus Necator (C.Necator)* DSM 545. For bio-based extraction, the organism *Bacillus Pumillus FH9* (Fcells) was used. 700g/L hydrolyzed sucrose (local brand "Fresh") was used as the carbon source. For the culture media, the mineral media composition used by [7]– (K_2HPO_4 = 7.0 g/L, KH_2PO_4 = 3.0 g/L, Sodium Citrate·$2H_2O$ = 0.5 g/L, $FeSO_4 \cdot 7H_2O$ = 0.05 g/L**)** was followed in this study, only changing the concentration of $MgSO_4$ to 0.8g/L. Variable amounts of Ammonium Sulphate, which has been showed to be the most suitable nitrogen source for enhancing the growth of *C. Necator* DSM 545 [8], was added to the growth media as the nitrogen source.

2.2. Inoculum preparation and reactor configuration

Stock cultures of *C. Necator* DSM 545 were stored in sterile agar plates (20g/L bacteriological grade agar, 10g/L LB medium) which were then used to inoculate growth media-containing flasks by adding necessary amount of substrate and swatches of bacterial colonies by using a sterilized loop. The liquid culture (i.e., the seed solution) was cultivated in a shaking incubator for 24-48 hours at 30˚C and 120 rpm. Sterile substrate was added into 100mL sterile growth media and then 5mL bacterial seed was inoculated into the growth medium under aseptic conditions. The pH was kept at 7 at all times (using pH meter: HI-8224 (HANNA Instruments, Romania). The reactors were then placed in the shaking incubator at 30˚C, 120 rpm. Samples were harvested and analyzed after specific intervals of time.

2.3. Analytical procedures

2.3.1. Measurement of optical density, glucose concentration, NH4-N concentration, dry cell weight

The extent of growth of *C. Necator* was tracked by measuring the optical density (OD). The concentration of glucose in the medium was determined off-line following the phenol-sulfuric acid method using glucose as a standard, as originally performed in [9]. The concentrations of ammonium-nitrogen (NH4-N) in the batch was determined by Nessler method, which uses spectrophotometry (UV-Vis Spectrophotometer: UV-1800 (SHIMADZU, Japan)). A gravimetrical method was used to determine the cell concentration, which was expressed as dry cell weight (DCW) in g/L. 15-20mL of the harvested sludge from the reactor was centrifuged in a centrifuge machine (Z287A, HERMLE Labortechnik Gmb, Germany), and the settled matter was dried overnight in an oven at 60˚C. The mass of dry biomass (measured using Analytical Balance: AS 120.R1 PLUS (Radwag, Poland), A&D Company, Limited (Japan)), per unit volume was noted as the dry cell weight (in g/L).

2.3.2. Extraction of PHB and PHB analysis

Extraction was performed using two methods—solvent-based and bio-based extraction. Bio-based extraction process required the dried biomass sample to be mixed with chloroform, followed by filtration to remove the undissolved cell debris. Finally, cold ethanol was used to separate out the pure polymer. Bio-based extraction was carried out according to the patent [10]. The bacterial strain of *Bacillus Pumillus FH9* (Fcells) was used as a cell-lytic agent to release the PHB from the other PHB-producing cells. For the PHB analysis, dried samples were analyzed in a thermogravimetric analyzer (TGA). The thermal degradation temperature and the PHB % was determined from the TGA curves. Extracted polymers were analyzed using Dynamic Scanning Calorimetry (DSC) to evaluate the melting temperatures, crystallization temperature, specific enthalpy of melting of the sample. Out of the total cell concentration, the PHB concentration (in g/L) was calculated from the PHB % obtained from the TGA data.

3. Results and discussion

3.1. Growth kinetics and PHA production behavior of *C.Necator* DSM 545

The total biomass growth (expressed as Dry Cell Weight), PHB accumulation, Optical Density, Cell Mass, PHB % together with the concentration profile of Ammonium-Nitrogen (NH4-N) using tap-water (from SUST) as the water source are shown in figure above. Optical Density, PHB % and PHB content increases with time. Also, at initially high concentrations of NH4-N available, the Cell Mass increases rapidly. The PHB content and PHB% also increases. A maximum DCW 15.69g/L, corresponding to a PHB percentage of 63.6% and a PHB content of 9.98g/L is achieved after 72 hours of growth time. The NH4-N concentration almost reaches 0 ppm at this point.

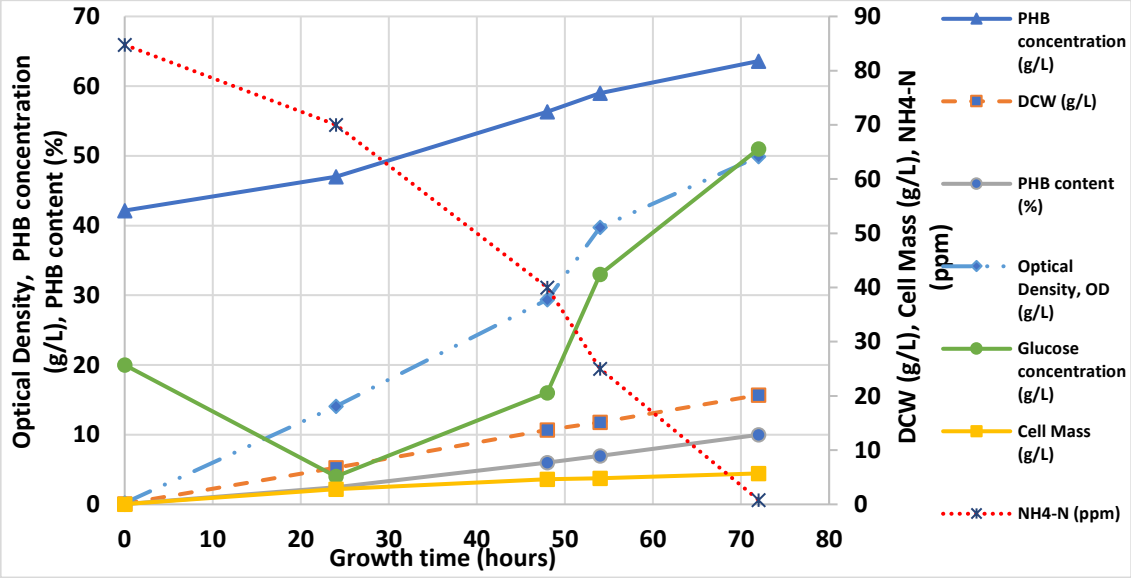


Fig. 1. Total biomass growth (as DCW), PHB content, Optical Density, Cell Mass, PHB content along with the concentration profile of Ammonium-Nitrogen (NH4-N) and glucose concentration.

Fig.1 indicates that there is a sharp increase in the Optical Density, PHB % and PHB content after the 24-hour mark is crossed. This is the point where nitrogen limitation comes into effect. The process of PHB production consists of a growth phase where cells grow under optimum growth conditions to yield a high cell concentration. This is followed by a polymer production phase under controlled growth conditions involving limitation of a nutritional component, such as nitrogen, oxygen, or phosphate, to induce PHB synthesis and accumulation [11]. When sufficient nitrogen concentration is available, biomass production predominates over PHB production, hence lower PHA content is observed. However, during nitrogen depletion conditions, PHA synthesis and accumulation occurs, hence cell mass decreases.

3.2. Effect of water source and initial nitrogen concentration

Experiment was conducted using different water sources and effect of initial NH4-N concentration was checked by evaluating the biomass harvested at the end of each experimental batch.

Table. 1. Maximum DCW, PHB concentration and PHB content obtained for different water sources.

Water Source	Initial NH4-N concentration (g/L)	Maximum DCW (g/L)	Maximum PHB concentration (g/L)	Maximum PHB content (%)
RO Water	2	8.01	4.81	60.00
	3	9.69	-	-
Commercial Drinking Water	2	13.07	-	-
	3	17.5	-	-
Tap-water	3	13.67	8.13	59.5
	4	15.69	9.98	63.6
Trace Element Solution aided-growth media	3	14.38	9.74	67.73

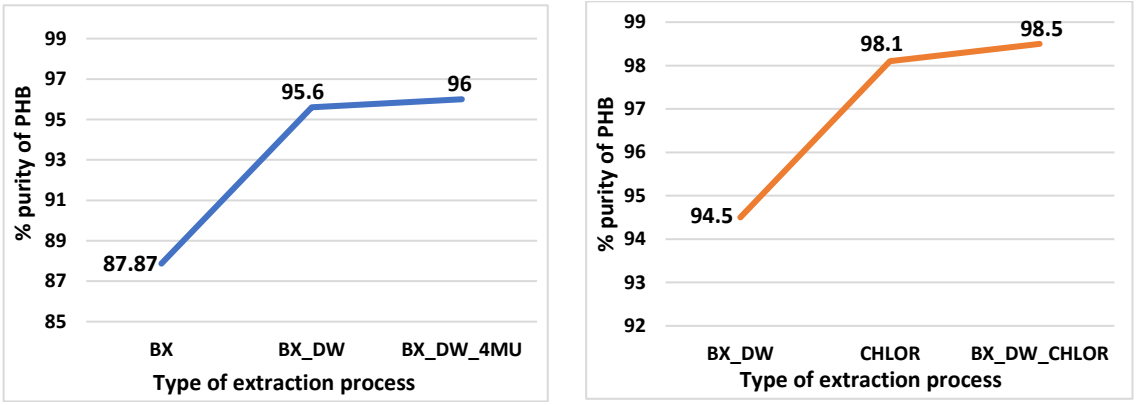
	4	14.77	9.59	64.96

As observed from **Table.1**, in each case, a higher initial NH4-N concentration resulted in greater maximum DCW, PHB concentration and PHB content. Among the 4 water sources used, RO-water resulted in the lowest final DCW of 9.69g/L over the span of 72 hours of fermentation while commercial drinking water resulted in the maximum achievable dry cell weight of 17.50g/L.

While Tap-water and TES-solution containing water also resulted in high enough DCW, preparing TES element solution is difficult, expensive and it needs to be stored carefully, which also creates added hassle and cost. Moreover, the concentration of ions and impurities cannot be controlled in Tap-water which may deter growth of microorganisms to begin with. Hence, tap-water was avoided. Commercial drinking water was chosen as the best water source, with low enough cost and high enough purity for PHB production.

3.3. Extraction of polymer

The polymer extracted using bio-based extraction (BX) resulted in a powder-like structure, with a purity of 87.87%. Upon applying detergent wash (DW), the purity increased by 7.73%, and upon further purification by washing with 20mL of 4 Molar Urea (4MU) solution, the purity increased by a further 0.4%.



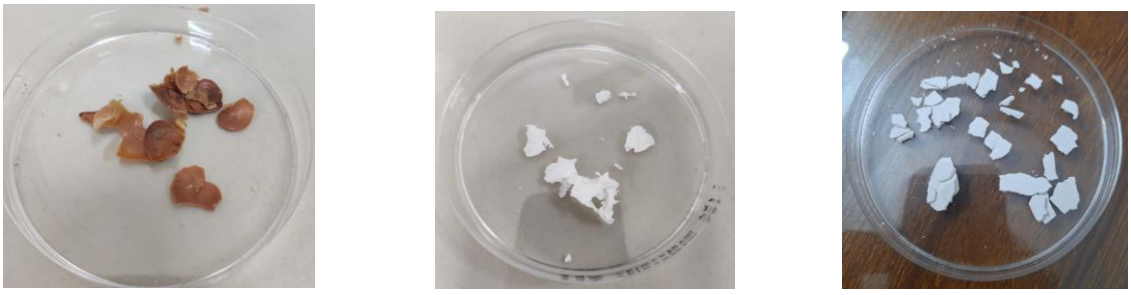
(a) **(b)**

Fig. 2. (a) Purity of PHB for different levels of bio-based extraction and **(b)** comparison in purity of PHB between solvent-based and bio-based extraction process. (BX: bio-based extraction, BX_DW: BX followed by detergent wash; BX_DW_4MU: BX_DW followed by washing with 4 Molar Urea solution; CHLOR: Chloroform extraction; BX_DW_CHLOR: BX_DW followed by chloroform extraction)

When a comparison was made between bio-based extraction and solvent extraction, the latter process resulted in a higher purity of the polymer. When the dried biomass sample was made to undergo bio-based extraction followed by detergent wash, 94.5% polymer purity was obtained and, as seen in **Fig. 2 (b)** above, if chloroform extraction only is carried out, the purity obtained is 3.6% greater. 98% purity is hence achieved by chloroform extraction only. If polymer obtained from bio-extraction followed by detergent wash is further made to undergo chloroform extraction, PHB% increases by 0.4%.

3.3.1. Appearance and polymer recovery of bio-based vs solvent-based extraction

Chloroform extraction resulted in a white, film-like polymer. Bio-based extraction resulted in a powder like polymer.



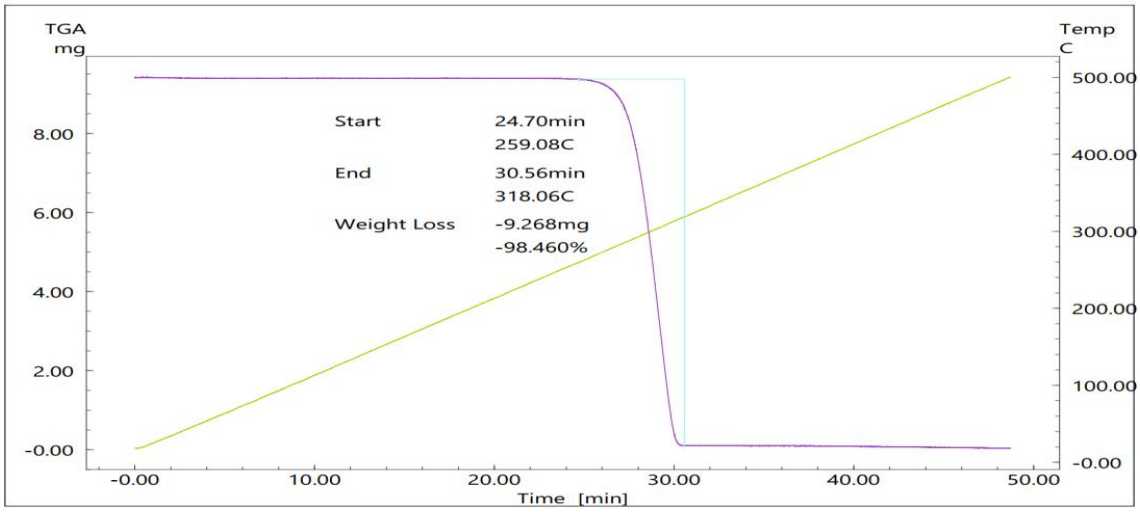
(a) **(b)** **(c)**

Fig. 3. (a) Dried biomass including PHB, **(b)** extracted polymer via chloroform, and **(c)** extracted polymer via bio-based extraction, for DSM 545 with hydrolyzed sucrose as substrate.

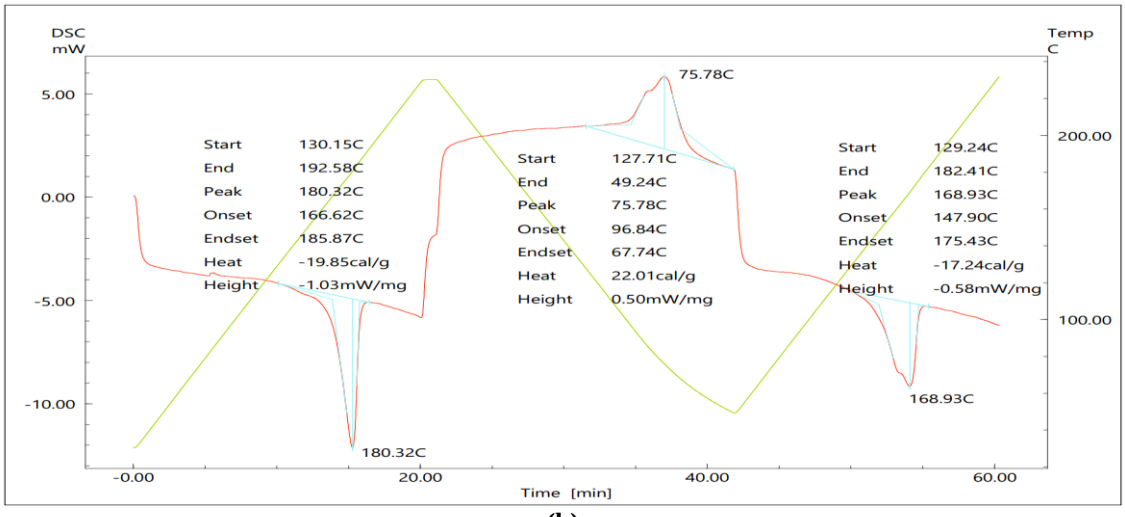
The post-extraction products indicated that chloroform extraction results in higher purity as well as film-like structure, whilst bio-based extraction results in lower purity and more powdery structure. The pictures shown in shown in **Fig. 3** also show that a higher recovery (gram polymer per gram biomass) was obtained using bio-based extraction than solvent extraction. In terms of polymer recovery, chloroform extraction resulted in a recovery of 61-64% whilst bio-based extraction yielded a higher recovery of 84%.

3.4. Thermal properties of extracted biopolymer: TGA and DSC results

Thermal properties of the samples were analyzed by Thermogravimetric Analyzer (TGA) and Dynamic Scanning Calorimetry (DSC). The TGA curves give the information about the thermal degradation temperature (T_{deg}) of polymer, and the weight loss % for complete polymeric degradation, which corresponds to the PHA %.



(a)



(b)

Fig. 4. (a) TGA and **(b)** DSC curve of extracted polymer sample.

In the **Fig. 4. (a)** above, a sharp dip in the weight of the biomass sample along with a sharp peak indicates that there is only one kind of monomer in the polymer produced. According to literature [12], the degradation temperatures and melting temperatures match the values for polyhydroxy-3-butyrate P(3HB). From the DSC curve, the first melting temperature (T_{m-1}), second melting temperature (T_{m-2}), crystallization temperature (T_c), and specific enthalpy of melting (ΔH_i) of the sample were noted for DSM 545. The Degree of Crystallinity, Cx, was calculated following the method mentioned in [13]. The thermal properties of the biopolymer produced are summarized in the table below.

Table. 2. The thermal properties of the biopolymer produced after extraction.

Substrate	Extraction Method	T_{deg}, (°C)	T_{m-1} (°C)	T_c (°C)	T_{m-2} (°C)	ΔH_i (cal/g)	ΔH_0 (cal/g)	Cx
Glucose	BX-DW	264	178.14	79.01	171.34	77	146	53%
Glucose	BX-DW-4MU	265	178.72	96.15	177.35	93.04	146	64%
Glucose	Chloroform	253	180.32	75.78	168.93	82.71	146	57%

4. Conclusion

- Using commercial drinking water can be a cost-effective alternative to laboratory grade RO-water and distilled water to produce Polyhydroxyalkanoates (PHAs).
- Higher initial nitrogen concentration enhances the microbial growth and PHA production.
- Using hydrolyzed sucrose as the substrate and sole carbon source, 70% PHA is achieved using DSM 545.
- Solvent-based extraction results in a lower recovery of polymer compared to bio-based extraction.
- Purity of polymer extracted by chloroform (98.5%) is higher than bio-based extraction (87.8%). Bio-based extraction followed by detergent washing increased the purity to 95%. Bio based extraction is a less toxic, more productive and more sustainable way of extraction.
- The melting temperatures and thermal degradation temperatures were found to be similar to previously found values for PHB.

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