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Production of Polyhydroxyalkanoates by *Bacillus megaterium*: Prospecting on Rice Hull and Residual Glycerol Potential

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Abstract: The production of polyhydroxyalkanoates (PHAs) by *Bacillus megaterium* using industrial residues, crude glycerol from biodiesel synthesis and rice hull hydrolysate (RHH), as low-cost carbon sources was investigated. The experiments were conducted by shaking flasks at 30 °C and 180 rpm up to 72 h. The extraction of PHA was carried out using sodium hypochlorite to make its recovery more environmentally friendly by avoiding organic solvents (chloroform). The yields of PHA varied depending on the extraction method. A total of 33.3% ($w \cdot w^{-1}$) (mixing chloroform: sodium hypochlorite) and 52.5% ($w \cdot w^{-1}$) (sodium hypochlorite only) were obtained using glycerol and glucose as a carbon source, respectively. Preliminary experiments using RHH as a carbon source Indicated a yield of PHA of 11% ($w \cdot w^{-1}$) (chloroform). The PHA produced had thermal properties, such as transition temperature, similar to the commercial polyhydroxybutyrate (PHB).

Keywords: PHA; biopolymers; residual glycerol; rice hull hydrolysate

Citation: de Oliveira Schmidt, V.K.; Santos, E.F.d.; de Oliveira, D.; Ayub, M.A.Z.; Cesca, K.; Cortivo, P.R.D.; de Andrade, C.J.; Hickert, L.R. Production of Polyhydroxyalkanoates by *Bacillus megaterium*: Prospecting on Rice Hull and Residual Glycerol Potential. *Biomass* **2022**, *2*, 412–425. https://doi.org/10.3390/biomass2040026

Academic Editors: Florent Allais and Lasse Rosendahl

Received: 21 October 2022
Accepted: 6 December 2022
Published: 16 December 2022

1. Introduction

The rising demand for petrochemical polymers and their improper disposal in the environment leads to serious problems, including the poisoning of wildlife, pollution of marine ecosystems, and emission of greenhouse gases, among others. It is, therefore, fundamental to develop alternatives to petrochemical polymers, ideally, polymers that are biodegradable and renewable [1]. In 2018, world production generated 359 million tons of plastic waste. In the last ten years alone, more plastics were produced than in the last century—the forecast is that it will double in the next 20 years [2]. In this sense, microbial biopolymers, in particular polyhydroxyalkanoates (PHAs), are remarkable compounds, since they are biodegradable, biocompatible, thermoplastic, and elastomeric polyesters. PHAs are promising alternatives to replace petrochemical polymers [3].

PHA is accumulated as intracellular granules in bacteria (Gram-positives and Gram-negatives) [4]. Bacteria such as *Cupriavidus necator*, *Bacillus cereus*, and *Bacillus subtilis* can accumulate up to 80% (cell dry weight) of their biomass as PHAs [5]. The production of microbial biopolymers is even more interesting when an alternative culture medium is used, for instance, agro-industrial wastes such as rice hull, wheat straw, sunflower seed hulls, cassava wastewater, and sugar cane bagasse, among others [6]. According to Dañez et al. (2020) [7], the use of corn straw, sugarcane bagasse, and banana pseudo-steam have equivalent potential to be used as alternative sources of substrate for polyhydroxybutyrate (PHB) production by *Bacillus megaterium*.

According to the United States Department of Agriculture (USDA), world rice production was estimated at 502.63 million tons for the years 2020 and 2021. Brazil stands out worldwide among the ten largest producers of rice [8]. In 2019, the Brazilian production of rice was ≈11 million tons, which represents approximately 2.2% out of world production [9]. The rice processing inherently generates 20% (mass fraction) of residues [10]. Similarly, the annual Brazilian production of biodiesel is ≈5.4 million m^3. It generates 440.6 thousand m^3 of glycerol (≈8.1% out of annual biodiesel production) as the main by-product [11]. It is worth noting that there is no report on PHA production by *B. megaterium* using rice hull as a low-cost culture medium. Thus, glycerol from the biodiesel industry can be a low-cost carbon source for many biotechnological processes, including the production of PHA [12,13].

The PHA production includes the screening of microorganism producers and their genetic engineering. In addition, its properties and applications depend on its monomeric composition, which, in turn, depends directly on the metabolic mechanism of the producing microorganism and the substrate used; that is, the production yields depend on the substrate used as a culture medium, which represents approximately 50% of total production costs [4].

In this sense, scientific trends were identified based on the research parameters (title, abstract, keywords, type of document, publication date, and patents) of the literature indexed in Scopus. The research was carried out on 11 October 2022, using the following keywords/booleans "rice hull" or "residual glycerol" and "biopolymers" or "production of biopolymers" or "PHA" or "*Bacillus megaterium*". After restricting the keywords most cited in this study to "rice hull" or "residual glycerol" and "*Bacillus megaterium*" or "PHA", 13 documents were found.

Based on the wide range of applications of these high value-added molecules and the lack of fundamental information, we studied the influence of different agro-industrial residues on the production of PHA by *Bacillus megaterium* in this work. Therefore, to reduce the production cost of PHAs and expand their application, the valorization of crude glycerol and rice hull hydrolysate is demonstrated.

2. Materials and Methods

2.1. Microorganism and Inoculum

Bacillus megaterium DSMZ32, a PHA producer, was acquired from the culture collection of Microbiology Culture Collection of BiotecLab (UFRGS, Brazil). It was kept on Petri dishes periodically replicated in LB medium and kept under refrigeration (4 °C). For the preparation of inocula (50 mL), the *B. megaterium* DSMZ32 was reactivated in a LB culture medium composed of yeast extract, 20 $g{\cdot}L^{-1}$, tryptone, 10 $g{\cdot}L^{-1}$, and sodium chloride, 10 $g{\cdot}L^{-1}$, supplemented with glucose (20 $g{\cdot}L^{-1}$) and ammonium sulfate (2 $g{\cdot}L^{-1}$). It was incubated at 30 °C, pH 7, and 180 rpm for 24 h on a rotary shaker. It was diluted with sterile saline solution (9%) to reach approximately 0.100 of absorbance at 600 nm. Then, 10% by volume was used as inoculum into Erlenmeyer flasks [14].

2.2. Culture Media

Three carbon sources were evaluated: glycerol from biodiesel production, rice hull hydrolysate (RHH), and glucose (control). Residual glycerol obtained through the transesterification of degummed soybean oil using methanol or sodium methylate in the reaction was kindly supplied by a soybean oil-based biodiesel manufacturing plant (Passo Fundo-RS/Brazil, centroid geo-coordinates at 28°15′40″ S and 52°24′30″ W). The composition of the residual glycerol was analyzed and contained (mass fraction): 81.8% glycerol, 5.82% ashes, 6.00% NaCl, 11.7% moisture, 0.6% monoacylglycerols, and pH 5.4 [15]. The rice hull was kindly supplied by local rice producers (Camaquã-RS/Brazil, centroid geo-coordinates at 30°51′04″ S and 51°48′44″ W, 39 m above sea level). The rice hull was dried and processed in a mill using a 0.8 mm sieve prior to acid hydrolysis. The RHH was prepared from 100 g of rice hull that was resuspended into 1 L (1% volume fraction)

aqueous sulfuric acid solution (98%). After sterilization (121 °C, 60 min), the hydrolysate was cooled in an ice bath and filtered using a cotton bag, and the permeate was adjusted to pH 7 with NaOH pellets. In order to increase sugar and protein concentrations, the hydrolysate was concentrated by evaporation at 70 °C [16]. Then, a basal mineral solution was elaborated composed of ($g \cdot L^{-1}$): $(NH_4)_2SO_4$ (2), $MgSO_4.7H_2O$ (0.008), Na_2HPO_4 (3.6), KH_2PO_4 (1.5), $FeSO_4.7H_2O$ (0.05), $CaCl_2.2H_2O$ (0.01), Citric acid (0.1), and 1 $mL \cdot L^{-1}$ of micronutrient solution composed of ($mg \cdot L^{-1}$): H_3BO_3 (300), $CoCl_2.6H_2O$ (200), $ZnSO_4.7H_2O$ (30), $MnCl_2.4H_2O$ (30), $(NH_4)_6Mo_7O_{24}.4H_2O$ (30), $NiSO_4.7H_2O$ (30), and $CuSO_4.5H_2O$ (10) [14].The three carbon sources tested were added to the basal mineral solution. The final concentrations of glucose was 12 $g \cdot L^{-1}$, residual glycerol was 50 $g \cdot L^{1}$ and RHH. Neither detoxification was made to the rice hull hydrolysate. Then, the culture media adjusted to pH 7 were autoclaved for 30 min at 120 °C at 0.5 atm [16].

2.3. Microbial Production of Polyhydroxyalkanoates

The sterilized culture media were individually inoculated with 10% (volume fraction) to a final working volume of 100 mL. Flasks were incubated at 30 °C and 180 rpm on a rotary shaker. Samples (5 mL) were collected every 12 h for 72 h [14].

2.4. Analytical Methods

2.4.1. Determination of Biomass

Microbial growth was followed by turbidimetry analysis at 600 nm using a UV/Visible spectrophotometer (Perkin Elmer model Lambda 265). The total biomass analysis was performed by gravimetry in which 3 mL samples were centrifuged at 1220× *g* for 30 min, and oven-dried at 80 °C [17]. The yield coefficient ($Y_{X/S}$) and volumetric productivity (P_x)—which is expressed as grams of product (X_m) per volume (L) times the final process time (t_f)—were calculated [18] according to Equations (1) and (2).

$$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f} \quad (1)$$

$$P_X = \frac{X_m}{L \cdot t_f} \quad (2)$$

The microbial growth of samples was expressed in colony-forming units (CFU/mL). Serial dilutions were carried out. Then 0.1 mL was inoculated on a solid Petri dish LB medium. The dishes were incubated at 37 °C for 24 h.

2.4.2. Quantification of Glucose, Total Sugars, and Glycerol

The quantification of glucose, total sugars, and glycerol was carried out by High Performance Liquid Chromatography (HPLC), (Shimadzu, Kyoto, Japan) equipped with a refractive index detector and Bio-Rad HPX-87H column (300 mm × 7.8 mm) using 5 mM sulfuric acid as an eluent at 45 °C, a flow rate of 0.6 $mL \cdot min^{-1}$, and a 20 µL sample. Samples were centrifuged (1220× *g*, at 25 °C for 30 min). The supernatant was diluted 1:10 and filtered with membrane pore size 0.22 µm (adapted Cortivo et al., 2018 [19]).

2.4.3. Extraction and Characterization of Polyhydroxyalkanoates

The polyhydroxyalkanoates were extracted using a solution of (1:1) chloroform (Synth) and sodium hypochlorite (12%, PROC9 industry). The extraction process of PHA was carried out at 37 °C for 1 h using 20 mL of solvent and 0.2 g of dry biomass [20]. Then, it was centrifuged at 1220× *g* at 25 °C for 30 min. Finally, the supernatant was oven-dried at 40 °C for 1 h [21], allowing polymer recovery in the form of a thin film. The obtained PHA was evaluated by Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) Spectrometry (Nicolet 6700, Thermo Scientific, Waltham, MA, USA) in the range of 4000–400 cm^{-1}. All measurements were taken from a sample surface in a temperature-controlled room on the built-in single-reflection diamond attenuated total reflectance (ATR)

crystal with a total of 64 scans. Differential scanning calorimetry (DSCQ2000, TA Instruments, New Castle, DE, USA) analyzes were also performed under a nitrogen atmosphere with a flow of 50 mL·min^{-1}. Approximately, 6.3000 mg of the sample were sealed in an aluminum planchet and analyzed. The samples were subjected to two cycles of heating/cooling at a rate of 20 °C.min^{-1} in the temperature range of −30 to 180 °C. The glass transition temperature (T_g), cold crystallization temperature (T_{cc}), and melting temperature (T_m) were obtained from the second heating cycle (20 °C to 180 °C) and crystallization temperature (T_c) in the first cooling cycle (20 °C to −30 °C). The degree of crystallinity (X_c) of the PHA was calculated (Equation (3)) by relating the melting enthalpy (ΔH_m) obtained through the endothermic peak with the value $\Delta H°_m$ of the 100% crystalline PHB (146 J·g^{-1}) [22]. The intracellular PHA fraction (% dry weight) was calculated using the capture value in grams of polymer from each extraction and total cells (X_r) used in the process in grams according to Equation (4) [14].

$$X_c = \frac{\Delta Hm}{\Delta H°m} \times 100 \quad (3)$$

$$\%_{PHA} = \frac{PHA}{X_r} \times 100 \quad (4)$$

2.5. Data Analysis

The data were analyzed using graphic software for data analysis and statistics, Thermal Advantage Universal Analysis (TA V4.5A) and OriginPro®9 (version 90E, Copyright® 1991–2013 OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Bacterial Growth Rate

3.1.1. Glucose and Residual Glycerol as a Carbon Source

The cell growth, substrate consumption, and biomass accumulation by *B. megaterium* over time are presented in Figure 1. In both cultures, values for cell biomass growing at 72 h, 3.88 ± 0.66 g·L^{-1} and 2.68 ± 0.61 g·L^{-1} for glucose and glycerol, respectively, were observed. The results suggest that kinetics were significantly different (Figure 1).

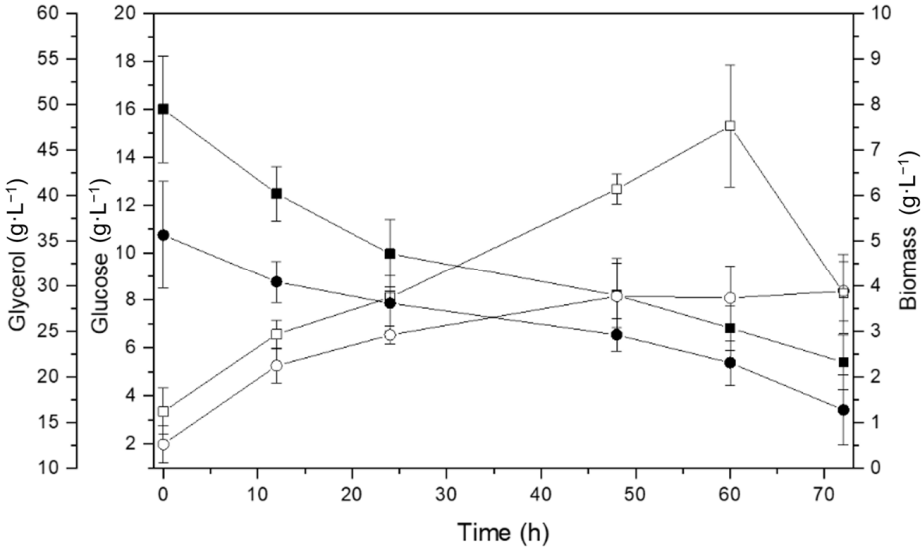

Figure 1. *Bacillus megaterium* DSMZ 32 kinetics. Glucose concentration (●), glycerol concentration (■), and biomass production using glucose (○) and glycerol (□) as substrate.

The PHA content was 1.19 ± 0.40 g·L^{-1} using glucose and 0.66 ± 0.38 g·L^{-1} with glycerol (Data from Table 1). When comparing glucose and glycerol as carbon sources for PHA production, the yield coefficient ($Y_{X/S}$)—glucose—was significantly higher. Consequently, glucose presented higher PHB content in dried weight. Table 1 shows the $Y_{X/S}$, $Y_{P/S}$, amount of PHA, and P_x of cultures. Even at a higher concentration (50 g·L^{-1} of glycerol), a lower production of PHA was observed (Table 1).

Table 1. Biomass yield per substrate ($Y_{X/S}$), product yield per substrate ($Y_{P/S}$), volumetric productivity (P_x), and concentration of PHA at 72 h.

Substrate	$Y_{X/S}$ (g·g^{-1})	$Y_{P/S}$ (g·g^{-1})	P_x (g·L^{-1}h^{-1})	PHA (g·L^{-1})
Glucose	0.52 ± * 0.18	0.18 ± 0.04	0.06 ± 0.00	1.19 ± 0.40
Glycerol	0.05 ± 0.07	0.02 ± 0.01	0.06 ± 0.00	0.66 ± 0.38

* ± standard deviation.

3.1.2. RHH as Alternative Carbon Source for the Polyhydroxyalkanoates Production

According to Hickert et al. (2013), factors such as processing technology, plant genetics, soil, and growing conditions can alter the chemical composition of rice hull. In the analysis of HPLC, the composition (% mass fraction, dry weight) of the rice hull used in this work was determined to be (g·L^{-1}): cellobiose (0.4), glucose (34.1), xylose (12.7), arabinose (1.3), acetic acid (1.3), 5-hydroxymethylfurfural (0.3), furfural (0.9), insoluble lignin (21.9), soluble lignin (6.1), extractives (3.1), ashes (15.9), and proteins (2.0). The preliminary tests using RHH as a carbon source indicated an unexpected difficulty to quantify biomass. It was initially proven that there were viable cells in the culture medium. Nevertheless, after 12 h, no colony-forming unit was observed, even at low dilution rates. PHA extraction using RHH was performed only by the conventional method (chloroform), producing an expressive amount (11% w·w^{-1}) when compared to other carbon sources. The analysis of HPLC indicated no consumption of complex sugars. After 72 h, biomass was collected by centrifugation, and the PHA was extracted by chloroform (Table 2).

Table 2. Percentage of PHA obtained through the three strategies used in the extraction methodology.

Solvent	Substrate	$\%_{PHA}$
Chloroform	Glucose	19.6 ± * 1.56
	Glycerol	22.3 ± 1.78
	RHH	11.0 ± 2.51
SH **	Glucose	52.5 ± 3.45
	Glycerol	16.0 ± 2.34
Chloroform:SH	Glucose	27.8 ± 5.75
	Glycerol	33.3 ± 2.24

* ±: standard deviation; ** SH: Sodium hypochlorite.

3.2. Extraction

Three PHA extraction strategies were performed: (I) chloroform (standard), (II) sodium hypochlorite, and (III) 1:1 chloroform and sodium hypochlorite. The analysis of Table 2 indicates that alternative extraction of PHA, when only sodium hypochlorite was used, recovered 52.5% (w·w^{-1}) of the polymer, using glucose as a substrate. The results were even more interesting using chloroform and sodium hypochlorite, simultaneously, since the culture medium did not affect it (27.8% glucose and 33.3% glycerol). The extraction with chloroform reached a PHA yield of 11 ± 2.51% (w·w^{-1}), using RHH as a substrate. It is important to note that only one strategy was used for this carbon source because, according to the literature, it presents more efficiency in extraction of PHA [21]. According to results obtained in this study, considering two substrates, the mixture proved to be promising. Moreover, the use of chloroform in extraction of PHA can be minimized or replaced by alternative solvents such as sodium hypochlorite.

3.3. Characterization of PHAs

3.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopic analysis was conducted to determine the functional groups of PHAs obtained by different carbon sources (Figure 2).

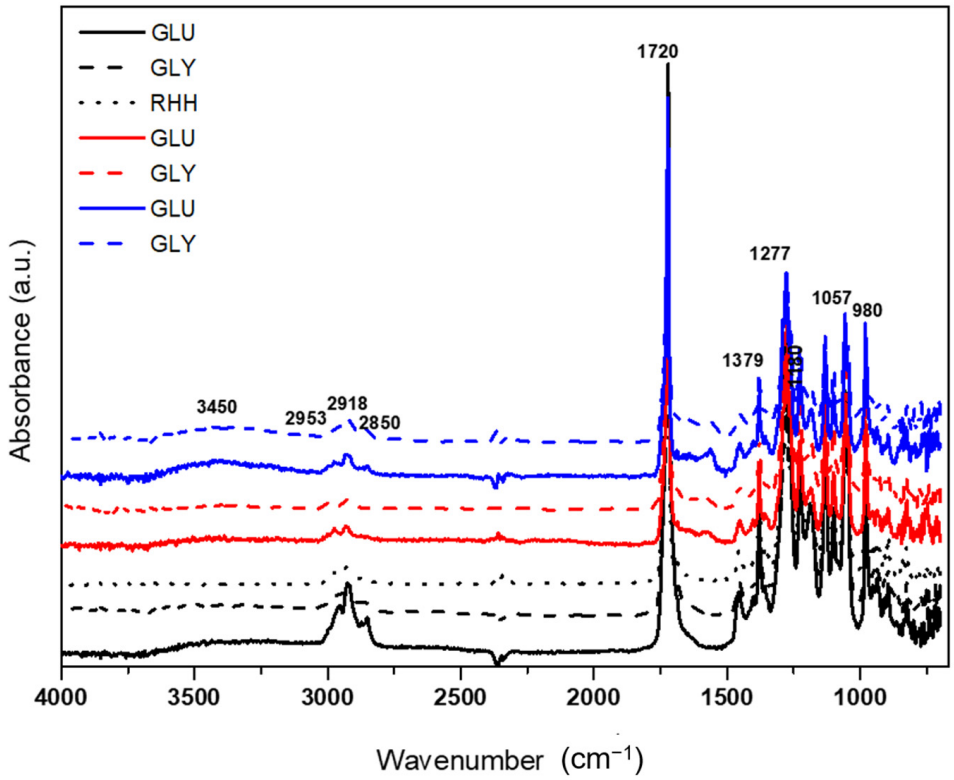

Figure 2. Spectrum of FTIR of PHA produced with glucose (GLU), crude glycerol (GLY), and rice hull hydrolysate (RHH) and extracted with chloroform (black), sodium hypochlorite (red), and with chloroform and sodium hypochlorite (blue).

FTIR spectra showed that typical bands of ester carbonyl (C=O) stretch around 1720 cm^{-1} and a number of bands, due to methyl (CH_3), methylene (CH_2), and ester (C-O) absorptions, between 1450 cm^{-1} and 800 cm^{-1}. These spectra are dominated by some characteristic absorbances for PHA, such as the bands at 1226 cm^{-1}, 1379 cm^{-1}, and 1280 cm^{-1} related to CH_2 and CH_3 deformations and C-O ester stretching, respectively. The stretch of bands ranging from 1054 to 1280 cm^{-1} showed C-O bonds of esters.

3.3.2. Differential Scanning Calorimetry (DSC)

Figure 3 shows the DSC of the first heating (Figure 3a), first cooling (Figure 3b) and subsequent heating scans (Figure 3c) of PHAs obtained with all substrates and recovered by different extraction methods. The relevant thermal parameters obtained from Figure 3 were listed in Table 3. Figure 3a shows that the PHAs obtained display several thermal transitions, such as glass transition temperature (T_g), cold crystallization (T_{cc}), and melting (T_m) temperatures during the first heating scan.

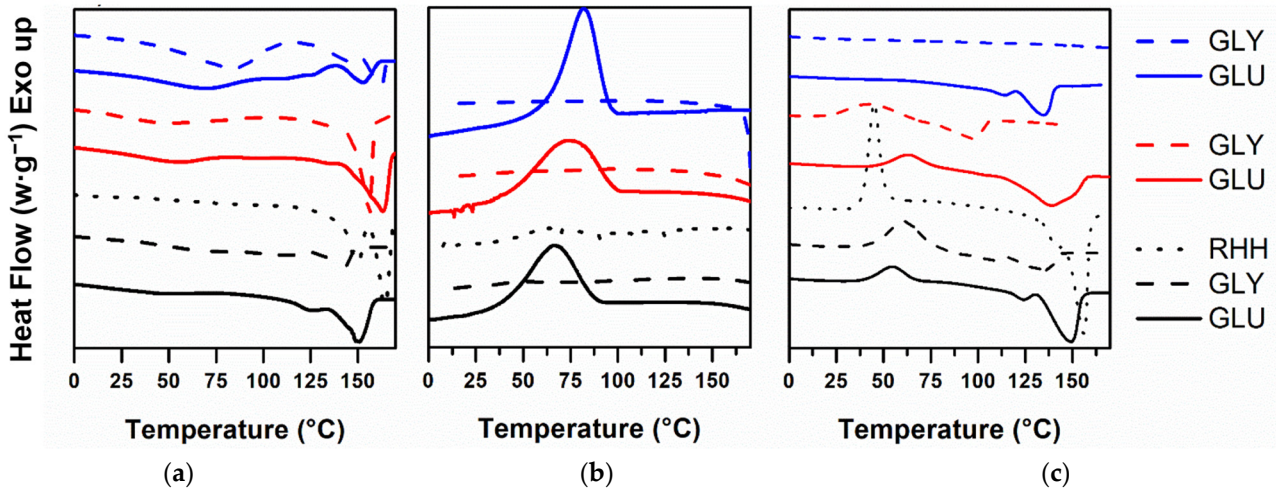

Figure 3. DSC (**a**) first heating (**b**) cooling and (**c**) second heating scans for the PHA produced with glucose (GLU), crude glycerol (GLY) and rice hull hydrolysate (RHH) and extracted with chloroform (black), sodium hypochlorite (red) and with chloroform and sodium hypochlorite (blue).

Table 3. Physical characteristics of PHA obtained by extraction with different solvents.

Carbon Source	Solvent	T_g *	T_c *	ΔH_c	T_{cc} *	ΔH_{cc}	T_m *	ΔH_m	X_c (%)
Glucose	Chloroform	−19	66	29.4	55	16.9	124–150	49.1	32
	SH ***	−10	73	37.8	64	15.8	139	56.3	33
	Chloroform:SH	−2	82	39.0	nd **	nd	126–147	49.8	34
Glycerol	Chloroform	nd	nd	29.4	55	16.9	107–138	18.5	13
	SH	−13	nd	nd	61	44.4	129	27.6	19
	Chloroform:SH	−14	nd	nd	nd	nd	nd	nd	0
RHH	Chloroform	−1	nd	nd	54	53	163	95	65

* T_g, T_c, T_{cc} and T_m: (°C); ΔH_c, ΔH_{cc} and ΔH_m: ($J \cdot g^{-1}$); ** nd: not detected; *** SH: Sodium hypochlorite.

4. Discussion

4.1. Bacterial Growth Rate

4.1.1. Glucose and Residual Glycerol as a Carbon Source

Gluconeogenesis is a metabolic pathway to synthesize glucose from carbon substrates without carbohydrates, such as glycerol [23]. According to Tanadchangsaeng and Yu (2012), glycerol is converted into the glycolytic intermediate dihydroxyacetone phosphate (DHAP),—part of the DHAP participates in the gluconeogenic phase via the metabolic pathway to produce precursors 4-, 5-, and 6-C, including glucose—which is first converted to phosphoenolpyruvate and then to pyruvate via glycolytic enzymes, completing the glycolytic pathway or via Embden Meyerhof–Parnas for the synthesis of the polymer and some metabolites (e.g., simple organic acids).

Moreover, gluconeogenesis is an essential part of the main metabolic pathway for the use of glycerol, and it can also cause less efficiency in the biopolymer biosynthesis rate and cell mass yield [24]. All conducted experiments resulted in a high amount of biomass associated with a low PHA production. These results are similar to that described by Figueiredo et al. (2014) [25], in which the accumulation rate of PHA was greater than the growth rate during exponential and stationary phases. Figueiredo et al. (2014) [25] used residual glycerol as an alternative fermentable substrate to observe time and temperature effects on PHA production by *Cupriavidus necator*, using an orbital shaker (150 rpm, at 30 °C and 24 h) and residual glycerol (30 $g \cdot L^{-1}$) and glucose (30 $g \cdot L^{-1}$) as a conventional

carbon source. They reported a maximum of 1.91 and 4.39 $g{\cdot}L^{-1}$ from glycerol and glucose for biomass production and 0.22 and 0.49 $g{\cdot}L^{-1}$ of PHA, respectively. After an increase in temperature and a longer fermentation time (72 h), an increase of PHA production was obtained (13.5%). Gómez-Cardozo et al. (2020) [26] evaluated PHB production using *Bacillus megaterium* LVN01. Batch and fed-batch cultures were used to produce PHB from residual glycerol. The maximum PHB productivity occurred at 30.8 °C and 39.9 h. The same conditions were tested in study and fed-batch culture. Fed-batch experiments were comparable to the batch one, where the cell dry weight was 1.9 $g{\cdot}L^{-1}$, and results in PHB productivities were 29.5 $mg{\cdot}L^{-1}h^{-1}$ and 35.6 $mg{\cdot}L^{-1}h^{-1}$ for bioreactors of 5 L and 14 L, respectively.

In our study, it is possible to observe (Figure 1) that the amount of biomass obtained using residual glycerol as a substrate in 24 h and 48 h of fermentation was higher (3.88 $g{\cdot}L^{-1}$ and 6.2 $g{\cdot}L^{-1}$) than the reported results by Cardozo et al. (2019) [27] as well as the productivity (60 $mg{\cdot}L^{-1}h^{-1}$). The glycerol was not totally metabolized due to the transport mechanism of *B. megaterium*—mainly diffusion (glycerol into cytosol) [28]. Additionally, crude glycerol is composed of toxic molecules such as methanol and NaCl that negatively affect microbial growth and PHA synthesis [29,30]. According to Mothes et al. (2007) in their study using residual glycerol containing 5.5% of NaCl, the PHB yield produced by *Cupriavidus necator* JMP 134 was reduced by 0.37 $g{\cdot}g^{-1}$ to 0.14 $g{\cdot}g^{-1}$, due to osmoregulation. On the other hand, glucose is easily metabolized by bacteria by the phosphotransferase system, phosphorylated during transport, and catabolized by glycolysis [14,31].

In order to use as primary carbon source, Pappalardo et al. (2014) [32] purified glycerol using physical-chemical methods: repeated cycles of acidification with H_2SO_4 (1.19 M). The acidification led to phase separation. Then, the phase rich in glycerol could be collected, neutralized with NaOH (12.5 M), and refined with ethanol. Thereafter, *Pseudomonas mediterranea* was cultivated for 72 h using the refined glycerol as a carbon source. The authors indicated that 3.0 $g{\cdot}L^{-1}$ of cell mass was reached. *C. necator* can also use glycerol as a carbon source for the production of PHB [33,34].

According to de Oliveira Schmidt et al. (2021), other efficient strategy to improve yields from biotechnological processes is the use of hydrophilic (e.g., glucose and glycerol) or hydrophobic inducers (e.g., hydrocarbons derived from petroleum and vegetable oils). The inducers are defined as supplementary carbon sources; that is, they are at low concentration. The PHA production is strictly regulated by interaction among microbial producers, nutrients, and modes of operation. Hydrophobic inducers act as a secondary carbon source for microbial growth. On the other hand, hydrophilic inducers are not primary carbon sources; hydrophilic inducers are co-factors molecules that assist cell growth and, thereafter, biopolymer synthesis [35].

Thus, Sabapathy et al. (2019) [36] investigated the production of PHA by *Bacillus aerophilus* RSL-7 utilizing Bael (*Aegle marmelos*) whole fruit hydrolysate as low cost substrate. The hydrolysate was supplemented with different concentrations (1, 2, and 3%) of the carbon (glucose) source to analyze the rate of PHA production. According to these authors, *B. aerophilus* inoculated into hydrolysate medium without any nutritional supplementation produced about 1.27 ± 0.08 $g{\cdot}L^{-1}$ of PHA at 72 h of incubation with 12.5 ± 0.26 $g{\cdot}L^{-1}$ cell dry weight. However, the highest amount of PHA produced was observed at 2.47 $g{\cdot}L^{-1}$ with dry cell weight of 21.25 $g{\cdot}L^{-1}$ at 72 h of incubation with 1% glucose supplementation, respectively.

Gourlate (2018) [37] using a parental strain and a recombinant strain of *C. necator* (glpFK) associated two substrates in different types of batch cultivation. The 24 h batch culture using 20 $g{\cdot}L^{-1}$ of glucose and 10 $g{\cdot}L^{-1}$ of glycerol showed better performance, while the cultivation of 20 $g{\cdot}L^{-1}$ of glucose and 25 $g{\cdot}L^{-1}$ of glycerol was the best percentage of final accumulation of PHB among the versions tested for culture. In an attempt to reduce the costs of the PHB production process and obtain greater performance in the process, an association of these substrates in fed-batch was also tested, in which it was the greatest

evidence of PHB using glucose as a co-substrate; no glycerol was added gradually after the glucose exhaustion. Similarly, Cavalheiro et al. (2009) [38] described the productivity of PHB between 0.6 and 1.5 $g{\cdot}L^{-1}h^{-1}$ by bioprocess with a high cellular concentration of *C. necator* DSM545 and glycerol as a carbon source. Naranjo et al. (2013) [39] compared the productivity and yield of PHB production by *B. megaterium* using glucose and analytical grade glycerol as carbon sources followed by the extraction with (1:1) chloroform:sodium hypochlorite. The authors indicated that the total cost of production of PHB can be reduced ≈15% by replacing glucose with glycerol (culture medium).

Additional research on the use of hydrophobic inducers associated with alternative carbon sources for production of PHAs has been reported. Sharma et al. (2015) [40] used *Pseudomonas chlororaphis* isolated from soya roots (native strain) in mixed canola oil with octanoic and nonanoic acid-free fatty acids like substrate to increase PHA production. Compared to a native strain, the production of PHA by *P. chlororaphis* PA23-63 modified was higher, up to 2.42–5.14 $g{\cdot}L^{-1}$ of cell biomass, and accumulated PHAs of 11.7–32.5% of cell dry weight when grown with octanoic acid, nonanoic acid, fresh canola oil, residual oil from canola fryer or fatty acids free of residues derived from biodiesel under batch culture conditions, respectively. With the results obtained, they can conclude that the addition of octanoic acid and nonanoic acid to canola oil cultures increased the production of PHA, but the addition of glucose not. The yields obtained by residual glycerol differ, like expected, compared to the production of PHA from glucose; the results confirm the ability of *B. megaterium* to use residual glycerol as a carbon source. The use of techniques to purify residual glycerol as well as the use of inducers can easily increase yields of PHAs. However, further investigation should elucidate its correlation with the metabolic pathways involved in this production.

4.1.2. RHH as Alternative Carbon Source for the Polyhydroxyalkanoates Production

Very likely, the bacterial growth was repressed by the high content of inhibitory compounds (5-hydroxymethylfurfural, furfural, and acetic acid) due to the sterilization [16]. The bacterial growth in a complex medium such as RHH requires an advanced metabolic apparatus, due to the complex molecules (e.g., protein, complex carbohydrates, etc), inhibitors, unbalanced carbon/nitrogen rate [41], and also osmotic pressure. The osmotic pressure of the hydrolysate medium is approximately 1539 $mOsm\ kg^{-1}$, significantly higher when compared to a synthetic medium composed of glucose (316 $mOsm\ kg^{-1}$) [16].

In addition, RHH induced *B. megaterium* to sporulate [42]. Other authors have also evidenced the low accumulation of biomass using hydrolysates as a carbon source. Yu and Stahl (2008) [43] reached only 2.73 $g{\cdot}L^{-1}$ of PHB using sugar cane bagasse hydrolysate. Methods for detoxifying hydrolysates become effective in this case and eliminate inhibitory compounds [44]. Kucera et al. (2017) [45] compared the use of activated carbon and lignite in detoxification of wood hydrolysate. Both methods of detoxification had a positive influence on *Burkholderia cepacia* and *Burkholderia sacchari* biomass. They obtained PHB yields of 8 to 12 times higher compared with non-detoxified hydrolysate. On the other hand, the authors report that the detoxification process was time-consuming and particularly expensive. Therefore, high dilution rates (hydrolysate), high initial cell density, high tolerant strain, methods of detoxification, etc. should be investigated.

4.2. Extraction

According to Jiang et al. (2006) [46], the extraction of PHA is usually performed by solvents, and the use of halogenated hydrocarbons such as chloroform is one of the most-used techniques. Chloroform (I) can change the permeability of the cell membrane by increasing the solubilization of PHA. Then, the phase containing PHA can be separated and evaporated [21], whereas sodium hypochlorite (II) can assist the chemical digestion of biomass, releasing the PHA granules [43,47,48]. Thus, chloroform and sodium hypochlorite can be simultaneously used for the extraction of PHA—strategy III, in which a three-phase system can be observed: chloroform, residual biomass (cellular debris), and sodium hypochlorite

(precipitated). In addition, there are some advantages in strategy III (chloroform and sodium hypochlorite), due to its high efficiency and simplicity, that contribute to obtaining higher recovery yields [21,49–51].

4.3. Characterization of PHA

4.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

Table 4 compares some physical properties and absorption bands in the infrared spectrum of the PHB (commercial and cited in the literature) and the PHA obtained in this study.

Table 4. Characteristic bands in the infrared spectrum and main physical properties of PHB and PHA obtained in this study.

Assignment of Bands	PHB *	PHA **
Axial deformation of the connection C=O (cm^{-1})	1735–1721	1721
Axial deformation of the connection C–C (cm^{-1})	978	978
Binding stretch C–H (cm^{-1})	2972–2850	2925
Asymmetrical and symmetrical stretching of the group C–O–C (cm^{-1})	1272 and 1058	1262 and 1055
Symmetrical angular deformation in the plane of the groups CH_3 (cm^{-1})	1380	1379
Physical Properties		
Melting temperature (°C)	175	163
Glass transition temperature (°C)	5	−1
Degree of crystallinity (%)	55–80	65

* PHB data obtained from the literature (Barud et al. (2011) and commercial (https://www.sigmaaldrich.com, accessed on 10 August 2022). ** PHA obtained in this study.

In general, the obtained biopolymers presented a broad and discreet band between the region 3100–3500 cm^{-1}, representing OH stretching vibration formed by hydroxyl groups between the polymer and water involved in hydrogen bonding. However, the higher intensity in samples extracted with the mixture, which can be associated with short-chain length polymers recovered [52,53] is evident. Analysis of the FTIR spectra could also provide additional information regarding differences in the chemical composition of the individual polymer materials. The comparison in characteristic absorption of alkyl groups showed clear differences in relative intensity around the region of 3000 cm^{-1}. This was noted mainly from the varying intensity of methyl and methylene absorptions at around 2872 and 2848 cm^{-1}, respectively). Nevertheless, further analysis could be done to provide additional information regarding the degree of structural components in the tested PHA materials. The major intense vibrational bands observed were also comparable with the peaks of PHA as reported earlier [27].

Figure 2 clearly illustrated the same absorption bands in the region of stretching frequencies (4000–1400 cm^{-1}), indicating that PHAs obtained from different carbon sources contain the same functional groups in their structure. Another observation is that the fingerprint region, characteristic of the compound as a whole, also showed a very similar pattern for all PHA samples. Thus, FTIR analysis provided a correct insight into the chemical structure of obtained polymers, reflecting the monomeric units present in PHA. The utilization of FTIR spectroscopy confirmed that the carbon alternative sources were able to produce PHA.

4.3.2. Differential Scanning Calorimetry (DSC)

According to Tripathi et al. (2019) the T_g of PHB is approximately (5 °C). The analysis of curves indicates that the T_g of PHA was lower than that presented by the literature. In addition to T_g, the samples showed an exothermic peak from the cold crystallization

process (T_{cc}) and an endothermic melting peak. In the analysis conditions, crystallization occurs partially during the cooling stage (from the melt) and partially during the reheating stage (as cold crystallization). The samples had different properties, and only the sample synthesized with the glucose source and extracted by the mixture was totally amorphous. The melting range of the polymers obtained occurred at temperatures much lower than the reference value for PHA of 173–180 °C [54]. The decrease in T_m associated with a broadened melting endotherm of samples can also indicate different monomer compositions in PHA. Additionally, the presence of two melting endotherms is characteristic of different compositions that result in different types of crystallites.

Another interpretation of the double melting has been given by Muneer et al. (2020). The authors reported that most multiple peak profiles are due to re-crystallization during melting. During cooling, only three samples showed the exothermic peak of crystallization (T_c). For these samples, the high cooling rate under experimental conditions tested does not have enough time to enable crystallization in the cooling cycle. The RHH based polymer showed a well-defined T_g at −1 °C, an exothermic peak of cold crystallization at 54 °C, and a melting peak at 163 °C. Interestingly, the analysis of DSC data indicated that the produced PHA from hydrolysate showed thermal properties more similar to commercial PHB (Table 4), with a sharp melting endotherm occurring at high temperatures.

We can see in Table 4, that the crystallinity values obtained for the extracted PHA samples using different solvents were found to be lower than that of commercial PHB, indicating the dependency of the crystallinity of the extracted PHA on the employed solvent. The degree of crystallinity obtained indicates that the corresponding PHB is a rigid and brittle material [53,55]. Nevertheless, the characteristics may limit its use in some end applications that require high impact strength.

5. Conclusions

In summary, PHA was produced by *B. megaterium* using glucose or crude glycerol-carbon sources. The microbial growth kinetics (glucose and crude glycerol) at 72 h were significantly different. Glucose is the most fermentable carbon source; nevertheless, the PHA total cost can be reduced ≈15% by replacing glucose with crude glycerol (culture medium). The present study revealed that RHH can be considered a promising alternative culture medium for the production of PHA. The optimization of conventional extraction methods was effective, with a PHA yield of 52.5% ($w \cdot w^{-1}$). The PHA obtained showed an exothermic peak of cold crystallization at 54 °C and a melting peak at 163 °C, respectively, with 65% crystallinity. However, inhibitors (sterilization), unbalanced carbon/nitrogen rate, and osmotic pressure should be carefully evaluated.

Author Contributions: Wrote the paper: V.K.d.O.S.; performed manuscript editing and revised the language: K.C., E.F.d.S., P.R.D.C., C.J.d.A., M.A.Z.A., D.d.O. and L.R.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge the State University of Rio Grande do Sul for the infrastructure to carry out the proposed work. We thank Marco Antônio Záchia Ayub for supplying the culture and structure of Bioteclab (Food Science and Technology Institute—Federal University of Rio Grande do Sul State) to carry out the analyses. We thank the technical team of the Multiuser Thermal Analysis Laboratory (Federal University of Rio Grande do Sul State), for their help in the characterization of the biopolymer.

Conflicts of Interest: The authors declare no conflict of interest.

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