

Article

Valorization of Dextrose from Cassava Starch and Sugarcane Vinasse as Polyhydroxyalkanoates by Submerged Cultures of *Cupriavidus necator*: A Physicochemical–Biotechnological Approach

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Abstract: The production of polyhydroxyalkanoates using submerged cultures of *Cupriavidus necator* DSM 428 was evaluated using low-cost substrates from agroindustry: (i) dextrose from cassava starch and (ii) a mixture of sugarcane vinasse from the bioethanol industry and dextrose from cassava starch. The effects of vinasse composition (2.5, 5.0, 7.5, 25, 50, and 75% *v/v*) and the use of raw and activated carbon-pre-treated vinasse were assessed. The results indicate that cultivations using only cassava starch dextrose reached 4.33 g/L of biomass as the dry cell weight and a poly(3-hydroxybutyrate) (PHB) production of 47.1%. Raw vinasse proportions of 25, 50, and 75% in the culture medium resulted in total inhibition. Vinasse treated at the same ratios led to biomass production in the range 1.7–4.44 g/L. The higher PHB production scenario was obtained in a medium containing dextrose and treated vinasse (7.5%), yielding 5.9 g/L of biomass and 51% of PHB accumulation. The produced PHB was characterized by XRD and FTIR for an analysis of crystalline structure and chemical functional groups, respectively. EDS was employed for a semi-quantitative analysis of the chemical composition, and SEM was used to analyze the morphology of the microgranules. The results of DSC and TGA analyses demonstrated the thermal stability of the obtained PHB.

Keywords: polyhydroxyalkanoates; cassava starch; sugarcane vinasse; *Cupriavidus necator*; low-cost substrates; mechanical testing



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1. Introduction

Over the last two decades, an increasing need has emerged to look for alternatives to petrochemical polymers that are more environmentally friendly. Currently, the use of conventional plastic is being replaced by other materials such as glass, steel, or silicone, and biodegradable bioplastics such as polyhydroxyalkanoates (PHAs), polylactic acid (PLA), and polybutylene succinate (PBS). PHA is the biopolymer that has stood out the most in recent years due to its high biodegradability, resistance, and versatility. Therefore, it is estimated that PHA could become a direct competitor against petrochemical plastics [1].

Poly(3-hydroxybutyrate) (PHB) is one of the most well-known and studied PHAs and is the only homopolymer in this family. PHB shares similar physical properties with polypropylene [2], including a similar melting point, crystallinity, molecular weight, and tensile strength. Concerning mechanical properties, PHB demonstrates high moisture resistance but is rather brittle and rigid. Nonetheless, due to its biodegradability, these properties may alter over time [3]. Furthermore, PHB is the most environmentally friendly

polymer, considering its biocompatibility and biodegradability. These characteristics enable its use as an implant material in the human body and as a carrier for the extended release of antibiotics [4]. These properties, combined with the homogeneous, dense, and nanostructured formation achieved through a biotechnology method, position PHB as a suitable biocompatible material for metallic implants.

According to Morlino et al. [5], *Cupriavidus necator*, formerly known as *Ralstonia eutropha*, is one of the most studied microorganisms for PHB production due to its versatile metabolic capacity. *C. necator* can grow both as a chemoautotroph and a heterotroph in aerobic and anaerobic environments, with the ability to utilize a variety of carbon sources for PHB synthesis. Currently, PHA production is limited by the high production costs, which are three to four times higher than those of synthetic polymers (between 0.60 and 0.87 USD/lb), hindering its industrialization and commercialization [6]. In the PHA production process, the carbon source could represent up to 50% of the final cost [7–10].

As reviewed by Wang et al. [11] and Bathia et al. [12], the use of waste as a carbon source in submerged cultures of *C. necator* for PHA production could be an alternative to reduce the final production costs. Currently, and over the last decade, many works are being carried out on the biosynthesis of PHA using various sugar-containing wastes as alternative substrates that allow for cost reductions [11,13–16].

Bitter cassava is one of the foremost prospective crops for added-value product generation in the bioeconomy, due to its high starch content (76.7% average [17]), low requirements for cultivation, and non-competition with food production [18]. Specifically, bitter cassava production in Colombia has grown significantly in recent years, with varieties characterized by high yields (25 ton/ha) becoming an important source of income for local farmers and contributing to the country's economic development [19]. Colombia produces about 269,000 tons of bitter cassava per year, used mainly in the food industry to produce glucose and fructose syrups, flour, concentrated cattle feed and bioethanol [18,20]. Therefore, the use of bitter cassava to obtain starch hydrolysate (e.g., dextrose), a potential carbon source, to produce high-value compounds such as PHA has been proposed.

Sugarcane exploitation is an established industry in Colombia, producing mainly table sugar for direct commercialization and the food industry, syrups for alcoholic fermentation, and bagasse for paper production and energy co-generation. Bioethanol distilleries produce between 2.2 and 3.1 million liters of vinasse daily [21,22]. Vinasse is a residual stream in distilleries characterized by a low pH, high chemical oxygen demand (COD) and biochemical oxygen demand (BOD), high dissolved organic matter content, as well as a considerable quantity of inorganic salts composed of chlorides, sulfates, phosphates, calcium, magnesium, and potassium [23]. Vinasse is potentially toxic because of its bio-recalcitrant substance contents, such as phenolic compounds and pigments like melanoidins, which can inhibit the activity of microorganisms [24]. Since vinasse production ranges between 11 and 15 liters for each liter of ethanol distilled [22], environmental concerns about its treatment and disposal have motivated the exploration of utilizing alternatives [23].

As a strategy to take advantage of the properties and micronutrients provided by both substrates, it is not unreasonable to use a combination of dextrose from cassava starch and sugarcane vinasse as a carbon source for *C. necator* cultivation. Thus, the aim of this study was to evaluate different dextrose/vinasse ratios as a low-cost carbon source for PHA production by *C. necator*, including no vinasse supplementation. To date and based on a detailed review of the scientific literature, this low-cost substrate combination has not been reported as a prospective alternative to bioplastic production.

2. Materials and Methods

2.1. Treatment and Characterization of Raw Materials Used as a Substrate

The carbon sources evaluated in this study consisted of solid dextrose from bitter cassava starch produced in the northern region of Colombia, and sugarcane vinasse from a local distillery in the southwestern region. Both substrates were donated by local industries.

Raw and treated vinasse were tested to evaluate the effect of the organic load of sugarcane vinasse on *C. necator* growth and PHA production. In both cases, the vinasse was centrifuged at 8000 rpm for 7 min to remove particulate matter. Total polyphenols were determined using the Folin–Ciocalteu method in a UV–Vis Jasco V730 spectrophotometer at 760 nm [25]. The pH was determined potentiometrically and °Brix was obtained via refractometry.

Sugarcane vinasse was treated to remove the organic component, which can be toxic, and/or inhibit the growth of microorganisms [26], which are difficult to biodegrade [27]. An adsorption system was designed based on previous work in our laboratory. Figure 1 shows a schematic of the experimental setup, which consisted of a borosilicate glass batch adsorption column (5 cm internal diameter and 30 cm length). A fixed bed (25 g) of granular activated carbon (GAC) was placed at the bottom of the column and, to sustain the carbon bed, a cylindrical support was constructed with two metal meshes at the top and bottom of the GAC bed.

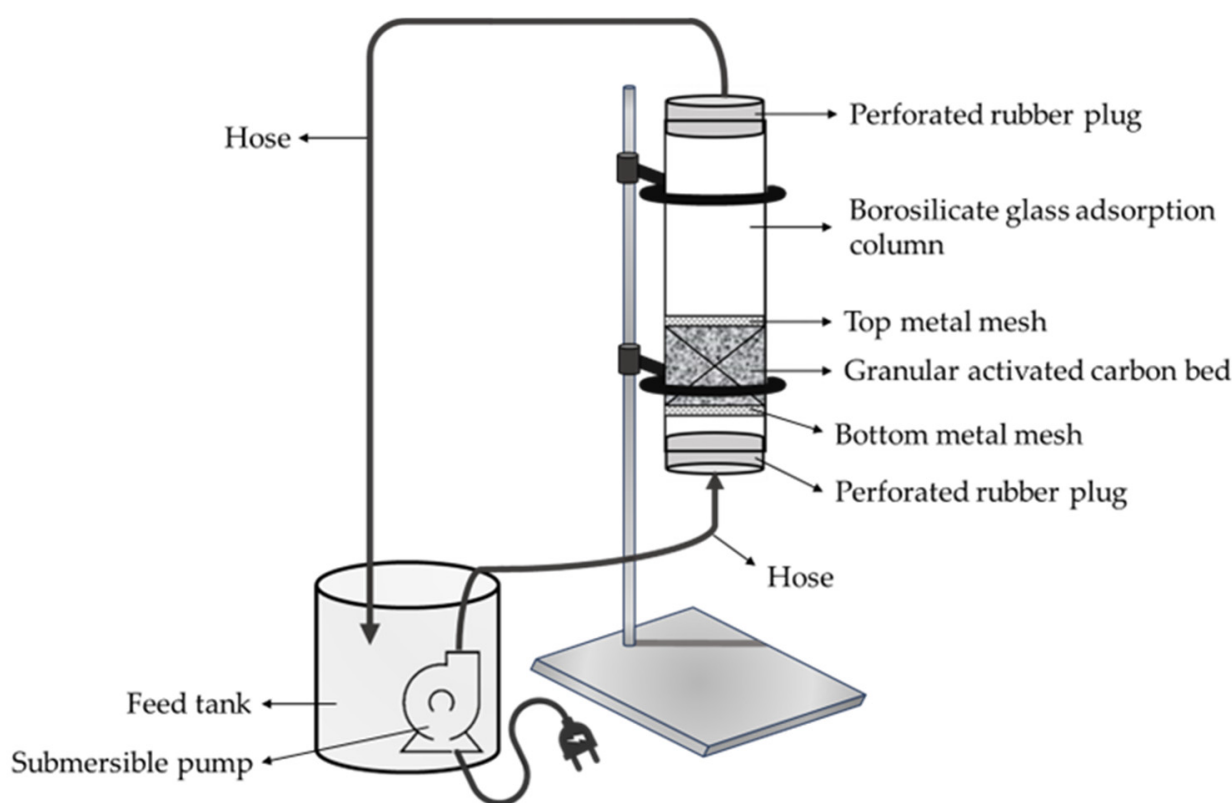


Figure 1. Schematic diagram of the experimental assembly used for the treatment of sugarcane vinasse.

The GAC used in this study was provided by a local supplier of water treatment solutions. The GAC was of bituminous origin and produced under strictly controlled conditions via high-temperature steam activation. This coal provides a large surface area, large pore volume, and an optimal pore structure for adsorption purification treatment. In accordance with AWWA Standard B 604-05 [28], the main physicochemical characteristics of the GAC are shown in Table 1.

Two treatment times for sugarcane vinasse were evaluated: 1.0 and 3.0 h. Since the polyphenols present in vinasse are one of the most relevant and concentrated inhibitors of biological activity, the removal of polyphenols was evaluated as an indicator of the treatment effectivity, and each test was performed at a constant up-flow (2.7 L/min).

Table 1. Physicochemical characteristics of granular activated carbon used as an adsorption agent for the treatment of sugarcane vinasse.

Parameter	Value
Iodine number	850 mg/g Min
Ash	15% Max
Humidity	5% Max
Harenes	90% Min
Granulometry	Mesh 8 × 30
Mesh8	5% Max/2 mm
Mesh30	5% Max/2 mm

2.2. Microorganism, Culture Media, and Cultivation Conditions

C. necator DSM 428 was obtained from the Leibniz Institute DSMZ collection (German Collection of Microorganisms and Cell Cultures GmbH) as a glass ampoule in a vacuum-packed double vial with a lyophilized tablet of a single strain of microorganisms. For its activation, the lyophilized cells from the ampoule were rehydrated and grown in 5 mL of liquid nutrient broth (NB). NB medium (PanReac AppliChem, ITW Reagents, Monza, Italy) was composed as follows: meat extract 3 g/L, meat peptone 5 g/L). The cultures were incubated at $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 24 to 48 h and the microbial growth obtained was aseptically added to a liquid medium composed of 20% glycerol and nutrient broth in 1.5 mL vials for subsequent storage at $-20\text{ }^{\circ}\text{C}$.

Initially, 1.5 mL of cryopreserved cells were reactivated in 50 mL of NB as seed medium, disposed in 250 mL shake flasks at $35\text{ }^{\circ}\text{C}$ and 150 rpm for 24 h. Two pre-culture stages were carried out for strain adaptation prior to production cultures. First pre-cultures contained 45 mL of modified mineral saline medium (MSM) and were inoculated with 5 mL of the cultivated seed broth. MSM medium contained (per liter) dextrose from bitter cassava starch 20 g, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 6.7 g, KH_2PO_4 1.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, iron and ammonium citrate 60 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 10 mg, and element trace solution 1 mL. Element trace solution (per liter): H_3BO_3 0.3 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 30 mg; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 30 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 20 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 10 mg [29].

Then, the second pre-cultures were prepared identically and inoculated with 5 mL of broth from first precultures. The PHB production cultures were performed in modified MSM medium, and using supplementation with treated and untreated sugarcane vinasse. Production cultures were inoculated at 10% *v/v* of second pre-cultures. and the cells were grown for 48 h at $35\text{ }^{\circ}\text{C}$ and 150 rpm. The pH was adjusted to 6.8 with NaOH 2M. Cultures were performed in triplicate. The composition of sugarcane vinasse varied between 2.5 and 75% *v/v* in each case. This variation aimed to evaluate the inhibiting effects of a wide range of sugarcane vinasse compositions in submerged cultures of *C. necator*.

2.3. Biomass and Sugars Quantification

Biomass concentration was quantified as cell dry weight in 1.0 mL samples. Samples were centrifuged at 5000 rpm for 10 min. Supernatants were used for sugar determination and wet cells were dried at $70\text{ }^{\circ}\text{C}$ for 24 h for further weight determination.

Reducing sugars were determined using the 3,5-Dinitrosalicylic acid (DNS) method [30].

2.4. PHA Detection and Extraction

The relative intracellular formation of the polymer was determined by lipophilic staining with Sudan black B, where the formation of PHA is detected along with the presence of black granules inside the cells. For the PHB extraction, the remaining cultures were centrifuged at 5000 rpm for 10 min. The obtained pellers were dissolved in 5 mL of sodium hypochlorite and 5 mL of chloroform. Then, cells were agitated in a vortex and kept at room temperature for 20 h in agitation, before being centrifuged at the aforementioned conditions. As a result, three separate phases were obtained: an inorganic phase (upper) corresponding to the sodium hypochlorite, the broken cellular material (middle), and the

organic phase of chloroform with the dissolved biopolymer (lower). The PHA present in each sample was precipitated by the addition of isopropyl alcohol. The precipitate was dried at 70 °C for 24 h and PHB dry weight was quantified [31].

2.5. Material Characterization

The crystallographic analysis of the PHB was performed using a panalytical X-ray diffractometer with Cu-K α radiation (wavelength $\lambda = 1.5405 \text{ \AA}$) operating at 45 kV and 40 mA. A parallel beam optical system was implemented, comprising a parabolic mirror in the incident beam, a 0.18° parallel plate collimator, and a flat graphite monochromator in the diffracted beam. X-ray scanning was conducted in the range of 5 to 90 degrees 2 θ , in step scanning mode, with increments of 0.03° (2 θ) and a counting time of 2 s. The functional groups and moieties present in the PHB powder samples were determined using Fourier transform infrared Spectroscopy (FTIR) (IR Affinity-1, Shimadzu Scientific Instruments, Columbia, MD, USA). The FTIR spectra were recorded in the range from 4000 to 500 cm⁻¹. For chemical composition evaluation, an energy-dispersive X-ray (EDS) analysis was performed using a Philips XL 30 FEG with a high-purity Ge EDS detector (Philips N.V, Eindhoven, The Netherlands). ZAF correction was applied to the stoichiometric analyses due to the low reliability of EDS under nitrogen concentrations. The structural analysis of the PHB was carried out via scanning electron microscopy (SEM) using a Philips XL 30 FEG, operating at 15 keV with a backscattered electron detector (EDAX-EDS). The thermal stability of the extracted PHB, along with standard samples, were analyzed via thermogravimetric analysis (TGA). The temperature range was from 30 °C to 600 °C, at a heating rate of 10 °C/min in a nitrogen atmosphere (N₂ flow rate = 40 mL/min). The degradation rate of PHB samples was performed using temperature data of T5%, T10%, and T50% obtained from the TGA analysis. The melting point (T_m) and glass transition temperature (T_g) of the PHB samples were determined via differential scanning calorimetry (DSC) analysis (DSC-1 series, Mettler-Toledo, Columbus, OH, USA) with a heating and cooling rate of 10 °C/min in a N₂ environment with a gas flow of 20 mL/min. For DSC analysis, 3.5 mg of the PHB sample was loaded in an aluminum pan and heated in the temperature range of -10 °C to 200 °C at a heating rate of 10 °C/min. The point of inflection in the DSC curve between onset and offset temperatures corresponds to the glass transition temperature and the melting point, measured as the peak temperature of an endothermic event.

3. Results and Discussion

3.1. Treatment and Characterization of Sugarcane Vinsasse

The raw sugarcane vinsasse exhibited high concentrations of polyphenolic compounds, in addition to high acidity (Table 2). The physicochemical parameters evaluated in the vinsasse exceeded the average values reported in the literature [26,27], which may be related to the concentration process used in distilleries.

Table 2. Physicochemical characterization of raw sugarcane vinsasse and polyphenolic compounds' removal in treated sugarcane vinsasse.

Parameter	Result	Treated Vinsasse	
		1 h	3 h
Total polyphenolic compounds (g/L)	18.39	18.39	15.31
Ashes (g/L)	93.19		
pH	4.88 ± 0.01		
°Brix	30.0 ± 0.1		

Table 2 also shows a comparison of the sugarcane vinsasse treatment using different adsorption times. As can be observed, for a time of 3 h, a reduction of 16.75% of the polyphenolic compounds present in the vinsasse was achieved.

Some studies have been reported that their presence can be toxic and/or inhibit the growth of certain microorganisms. However, there are reports indicating that no inhibitory effects are observed when vinasse is used as a carbon source [32]. Additionally, the high sugar content present in the vinasse makes it a viable substrate for the growth of *C. necator*.

In general, the chemical composition of vinasse is quite variable, depending on the quality of the juice, provenance, harvesting conditions, fermentation, and distillation process used. All these conditions represent challenges for the use of vinasse as a carbon source to produce PHA through microbial fermentation.

3.2. Kinetic Evaluation of *C. necator* in Dextrose Medium and PHB Production in Shake Flask Cultures

Figure 2 shows the evolution of glucose consumption, biomass, and PHB production over time in shake flask cultivations of *C. necator* using dextrose from cassava starch as a low-cost carbon source. The lag phase ended 12 h after culture started. Subsequently, an exponential phase was observed, ranging from 12 to 48 h of cultivation, followed by a stationary phase of 12 h. The maximum biomass concentration achieved was 5.17 g/L at 48 h. At the end of the cultivations, the glucose concentration was 10.75 g/L, which corresponds to 53.7% of the initial concentration. PHB production and accumulation was evaluated at the end of the cultures (72 h). PHB concentration was 2.01 ± 0.10 g/L, which corresponded to a PHB content of 47.1%. The accumulations of PHB content were visually confirmed through a microscopy utilizing the Sudan black staining method (see Figure 3), which was monitored after 24, 48, and 72 h of cultivation. This qualitative analysis allows for the observation of the progressive increase in polymer intracellular accumulation as cultivation advances.

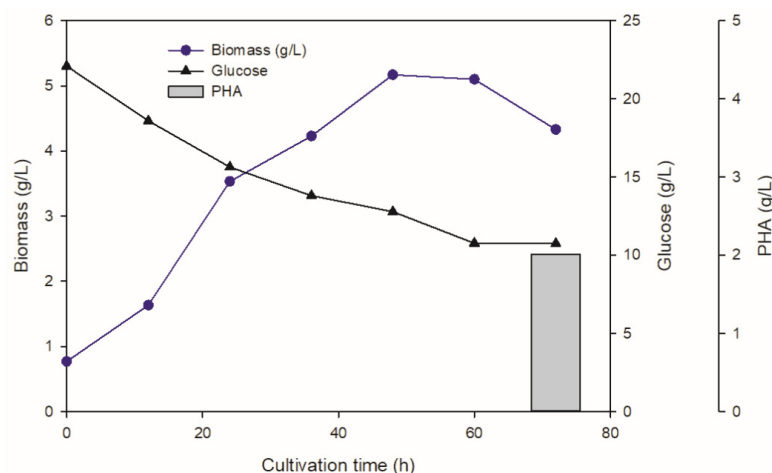


Figure 2. Glucose consumption, biomass, and PHB production over time in shake flask cultivations of *C. necator* using dextrose from cassava starch hydrolysate as a carbon source.

In this culture, the growth of *C. necator* in carbon sources rich in glucose, such as dextrose from cassava starch, was demonstrated. Additionally, it was observed that there was no depletion of the carbon source by the end of the culture. This behavior mirrors that reported by [28] when employing the same strain (*C. necator* DSM 428) in cultures with concentrations ranging from 5 to 20 g/L of glucose, where substantial amounts were reported at the end of the culture (between 1.4 g/L and 17.4 g/L). The same author reports that only with 2 g/L glucose was the growth phase limited by the total consumption of the carbon source. Other authors [33] reported a decrease in biomass quantity when the glucose concentration was 20 g/L, attributing this concentration to the inhibition of growth in *C. necator* DSM 545. Furthermore, they indicate that with glucose concentrations higher than 10 g/L, complete glucose depletion does not occur at the end of the cultivation. In our study, glucose concentrations of 20 g/L were utilized; however, the biomass production at 48 h exceeded the data reported by [33]. Despite the absence of glucose depletion in the

medium, it is likely that nitrogen source depletion (ammonium sulfate) occurred, enabling PHB accumulation.

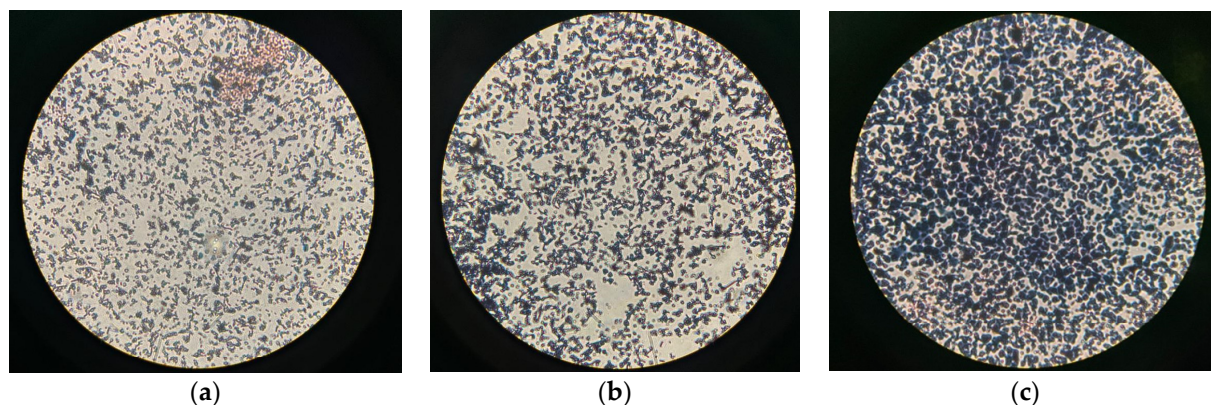


Figure 3. Detection of PHB by 100× microscopy using Sudan black staining: (a) 24 h, (b) 48 h, and (c) 72 h.

Furthermore, the production of PHB through the microbial cultivation of *C. necator* has been extensively studied, and other works reported the utilization of similar carbon sources for biopolymer production [15,34,35]. In this process, the PHB content is higher than that reported by [36] for *C. necator* after 58 h in culture in a study utilizing broken rice as a carbon source (38% PHB). Similarly, Oliveira et al. [37] reported a PHB accumulation of 33.3% when employing soybean and 2.5% molasses in solid-state fermentation with *C. necator*. Likewise, a study reported an accumulation of 42.2% at 31 h of cultivation of *C. necator* DSM 545 using fructose as the carbon source [38]. In contrast, higher accumulations (84.3 and 92%) were reported at 72 h of cultivation for strains *C. necator* IBP/SFU-1 and *C. necator* B-10646, respectively, using glucose as the carbon source [16,39]. Therefore, glucose is a promising carbon source for PHB production using *C. necator*. Even though fructose is the only sugar capable of being metabolized by hydrogen-oxidizing bacteria, they can easily mutate, enabling them to metabolize glucose through the Entner–Doudoroff pathway to form pyruvate, which is transformed by dehydrogenase into Acetyl-CoA, one of the precursors of PHA, as demonstrated in this study using *C. necator* DSM 428 [16,40].

3.3. Evaluation of *C. necator* Growth and PHB Production with Dextrose from Cassava Starch and Sugarcane Vinasse as a Supplement

The results of biomass and biopolymer accumulation obtained using vinasse supplementation and dextrose from cassava starch in different proportions are shown in Table 3. In addition, at the end of the production time (48 h), the presence of PHB granules in the cells was confirmed by staining with Sudan Black B and through microscopic observation, as displayed in Figure 4.

As observed in Table 3, the use of a medium with a raw vinasse composition of 25% *v/v* (and higher) totally inhibited *C. necator* growth. On the contrary, in media with a raw vinasse composition between 2.5 and 7.5% *v/v*, bacterial growth was observed.

A clear trend is observed in cultures with a raw and treated vinasse composition between 2.5 and 7.5% *v/v*: as the proportion of vinasse in the medium increases, bacterial growth increases, evidencing the nutritional effect of the organic assimilable compounds in the mixture, mainly carbohydrates. Despite no significant changes in the accumulation of the biopolymer being observed in this range of concentrations (2.5–7.5% *v/v*), more biomass was produced in each case, yielding a higher final PHA concentration. Moreover, for the most concentrated media with the treated vinasse (25, 50, 75% *v/v*), a lower PHB accumulation was attained.

As increasing values of nitrogen are provided to the culture by increasing the vinasse concentration, the addition of both nutrients, carbon and nitrogen favor cell growth but not the PHB accumulation, as shown in the results of Table 4.

Table 3. Experimental results of biomass produced and PHB accumulation under different proportions of sugarcane vinasse (raw and treated) in submerged cultures of *C. necator*.

Vinasse	Vinasse Proportion in Medium (v/v)	Biomass Concentration (g/L)	Polymer Concentration (g/L)	Polymer Accumulation (%)
Raw vinasse	2.5%	2.97 ± 0.13	1.43 ± 0.06	48%
	5.0%	3.72 ± 0.13	1.77 ± 0.07	48%
	7.5%	5.50 ± 0.13	2.66 ± 0.11	48%
	25.0%	No growth	No production	0%
	50.0%			
	75.0%			
Treated vinasse	2.5%	3.30 ± 0.14	1.57 ± 0.06	48%
	5.0%	3.87 ± 0.14	1.96 ± 0.08	51%
	7.5%	5.90 ± 0.14	2.98 ± 0.12	51%
	25.0%	1.70 ± 0.13	0.69 ± 0.03	41%
	50.0%	3.25 ± 0.13	1.43 ± 0.06	44%
	75.0%	4.44 ± 0.13	1.82 ± 0.08	41%

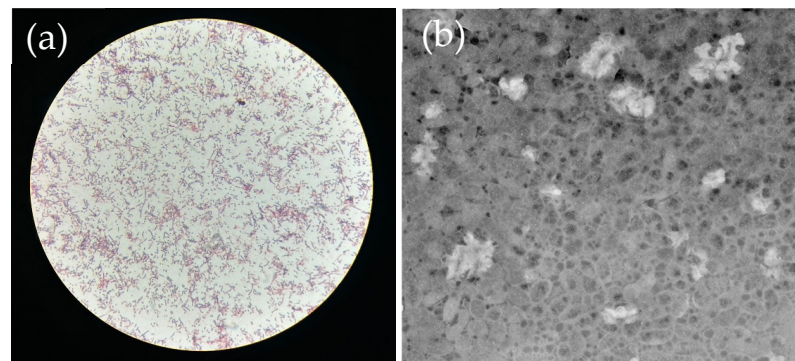


Figure 4. Intracellular accumulation of PHB in the bacterium *C. necator* in medium with treated sugarcane vinasse 7.5% v/v; (a) identification of PHB by staining with Black Sudan B (magnification 100 \times); (b) scanning electron micrographs (SEM), magnification 5000 \times .

Table 4. Chemical composition of the PHB produced by *C. necator* using dextrose from cassava starch as a carbon source.

Spectrum	In Stats.	C	O	Na	Cl	Total
Average	Yes	50.74	38.77	4.61	5.88	100.00

Additionally, when the treated vinasse proportion in the medium is 25% v/v, a considerable decrease in the amount of biomass obtained is seen. Nevertheless, when the proportion increases again between 50 and 75% v/v, biomass production tends to increase again, which can be justified because although the concentration of polyphenols in the medium increases, sugars are more available. Therefore, the increase in vinasse in the medium leads to an increase in compounds that have negative and positive effects that counteract each other.

Nevertheless, it is important to highlight the case in which treated vinasse was used in a proportion between 50 and 75%. Although a higher vinasse concentration does not imply that higher ratios of PHB accumulation will be achieved, the results are promising.

Significant cell growth was obtained with a high amount of vinasse using a treatment for a small remotion of inhibitors, reducing the need for water in the medium.

Therefore, in terms of resource utilization, the use of the medium with the highest amount of treated vinasse (75%) could be the best scenario from the industry standpoint. However, in terms of production, the results indicate that using treated vinasse at a proportion of 7.5% is the best option, which leads to a PHB production of 2.98 ± 0.12 g/L, which slightly exceeds the concentration reported in other studies with similar substrates [26], and a PHB accumulation of 51%. The above is a promising result that demonstrates that the treatment of sugarcane vinasse contributes significantly to the increase in PHB production, as investigations using the same microorganism and operating conditions, using raw vinasse without any treatment, led to lower biopolymer accumulations (between 26 and 33%) [32]. However, the results obtained may be better if the culture medium is supplemented with another carbon source, such as molasses, which is also a residue from ethanol distillation. Researchers evaluating different proportions of vinasse/molasses in a mineral medium reported accumulations from 56 [41,42] to 97% [26]. Likewise, accumulations of up to 76% are recorded when increasing the glucose concentration in the medium (50 g/L) [43].

Therefore, considering that one of the main disadvantages for the commercialization of PHA is its high production cost, up to three times higher than that of conventional plastics [44], the use of low-cost carbon sources, such as those studied in this work, could reduce this gap; it is reported that the use of vinasse could achieve a decrease in production cost of up to 22.2% [6].

Figure 5 shows the biomass and sugar time-courses of *C. necator* for the production medium supplemented with treated vinasse 7.5% *v/v* and the production medium with only dextrose.

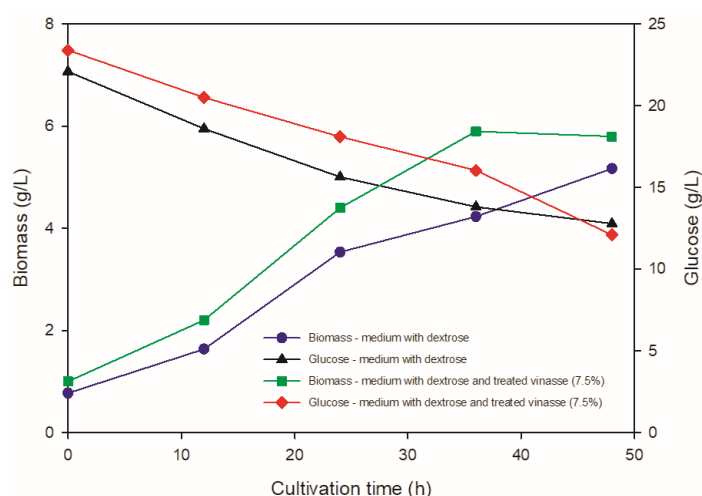


Figure 5. Mean values of biomass growth and glucose consumption by *C. necator* in culture media with dextrose and dextrose-treated sugarcane vinasse at concentration of 7.5%.

As can be observed in Figure 5, for culture supplemented with sugarcane vinasse, the exponential phase starts at 12 h and growth lasts up to about 36 h. Then, the stationary phase can be observed. The biomass accumulation was 5.80 ± 0.12 g/L at 48 h of culture. The remanent glucose at the end of the culture was 12.10 g/L, with a PHB accumulation of 51%. Similar trends were observed in the case of cultures with only dextrose, with a maximum biomass concentration of 5.17 ± 0.027 g/L. The glucose concentration at the end of the culture was 10.75 g/L, with a PHB accumulation of 47.1%.

According to the results obtained, the addition of vinasse to the medium had a positive impact on cell growth due to its supply of sugars (glucose, fructose, sucrose) and other nutrients, such as organic acids, as reported in the literature [45]. This contributed to a biomass accumulation during the exponential phase that was higher than that obtained

using carbon sources like dextrose [41]. However, comparing the percentages of PHB accumulation in both cultures, dextrose can be considered to be suitable as the sole carbon source, achieving a good percentage of this bioplastic.

Now, considering the processes involved in using vinasse as a carbon source, and to minimize production costs, the PHB obtained from dextrose was chosen to be physically and structurally characterized.

3.4. Biopolymer Characterization

3.4.1. XRD Analysis

Figure 6 presents the XRD pattern of the PHB obtained via the microbial cultivation of *C. necator* using dextrose from cassava starch. From these results, an Orthorhombic phase with a 19-P212121 space group belonging to PHB was evidenced by the reflections of the crystallographic planes: (020), (110), (130), (202), and (410), located at angles $2\theta = 13.52^\circ$, 16.91° , 27.28° , 31.62° , and 45.341° , respectively, indexed through the reference file JCPDF 00-001-0182. Similar XRD patterns of PHB were reported by [46,47]. The intense (202) peak indicates the crystalline nature and it has been proposed that the polymer matrix adopts a regular helicoidal conformation with two antiparallel chains in the Orthorhombic unit cell within the crystalline domain. In this sense, it is possible to observe the PHB obtained by *C. necator* with a unit cell, which consists of an orthorhombic crystalline structure system [47,48].

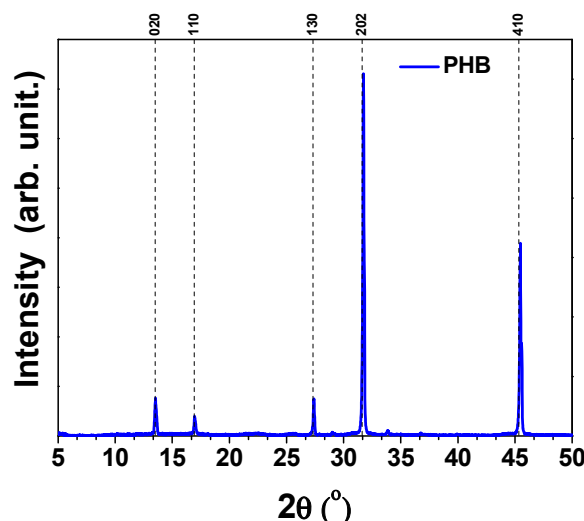


Figure 6. XRD of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source.

3.4.2. FTIR Spectroscopy

The vibration modes of the bonds present for the PHB were analyzed using the FTIR spectra presented in Figure 7. In addition, the FTIR spectra were fitted by Gaussian functions (deconvolutions) to analyze the internal signals present in the material. Figure 7 shows the FTIR spectrum belonging to PHB, where it was possible to determine four high-intensity bands located at 520 cm^{-1} , 1278 cm^{-1} , 1721 cm^{-1} , and 3442 cm^{-1} , respectively. On the one hand, those bands are associated with the vibrational states of the C=O, C=H, and CH₃ bonds of PHB obtained in our study [49,50]. On the other hand, it was possible to determine a bond corresponding to a wave number of 536.1 cm^{-1} , associated with the tensile vibrational state of C–H from the stretching methyl group, while the band at 1074.1 cm^{-1} corresponds to the ester bonding, and the band at 1271.8 cm^{-1} corresponds to the symmetric bending H–C–H from CH₃ groups. The 1721 cm^{-1} signal was associated with the C=O ester carbonyl group, 2947.8 cm^{-1} , and the band at 2947.8 cm^{-1} reveals the presence of alkyl (CH₃) halides, which confirms that the polymer is PHB [47,48]. Furthermore, the band at 3270.8 cm^{-1} corresponds to the asymmetrical deformation of

the C–H bond in the CH₂ group. Finally, a band located at 3450.1 cm^{−1} is attributed to the energy absorption caused by the stretching of the O–H bonds [51]. These results also coincided with those obtained by [37,38,47,52].

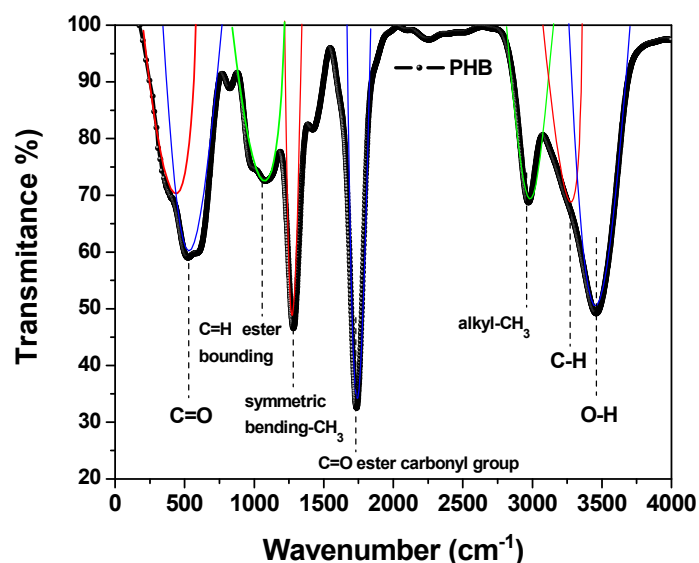


Figure 7. FTIR characterization of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source.

3.4.3. EDS Analysis

To investigate the chemical composition of the formed biopolymer, an EDS analysis was conducted on PHB synthesized by *C. necator* (see Figure 8). This analysis shows carbon and oxygen to be principal components, with 50.74 wt% and 38.77 wt%, respectively [53,54]. The results indicate a very low level of impurities, which correspond to the sodium and chlorine traces from the PHB extraction method employed in the current research (Table 4). Other studies reported residual elements such as sodium hypochlorite, an essential component during the extraction process [54,55]. To remove these types of impurities, PHB can undergo an effective purification process that enables its safe commercial application.

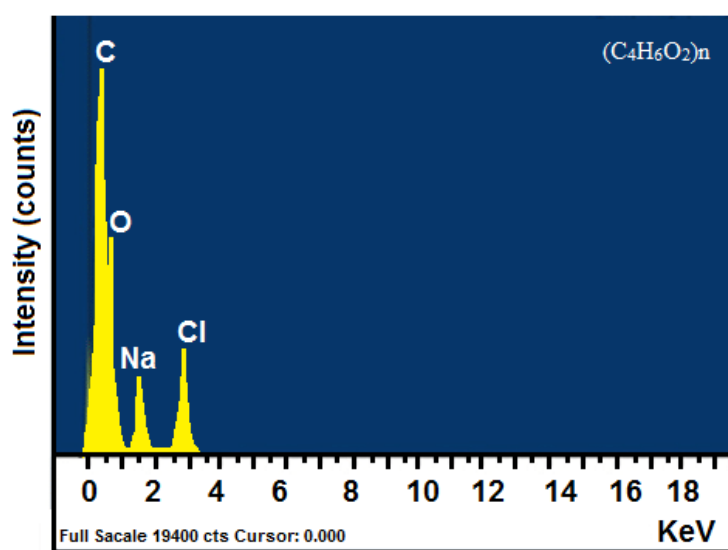


Figure 8. EDS characterization of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source.

The above is in accordance with the presence of sodium and chlorine in Figure 8.

3.4.4. SEM Analysis

The SEM micrograph analysis obtained for the PHB shows a distribution of micro- and nano-pellets with a spindle-shaped morphology (Figure 9a). In Figure 9b, the magnification of the obtained pellets is analyzed, showing an irregular surface with a varied morphological distribution from angular rounded geometries with flake shapes [53]. A similar semi-crystalline morphology with a brittle appearance characteristic of PHB was also reported by [55]. The morphological nature analyzed by SEM allows us to identify—from the morphology of the PBH pellets—that they are optimal for subsequent extrusion or 3D printing processes.

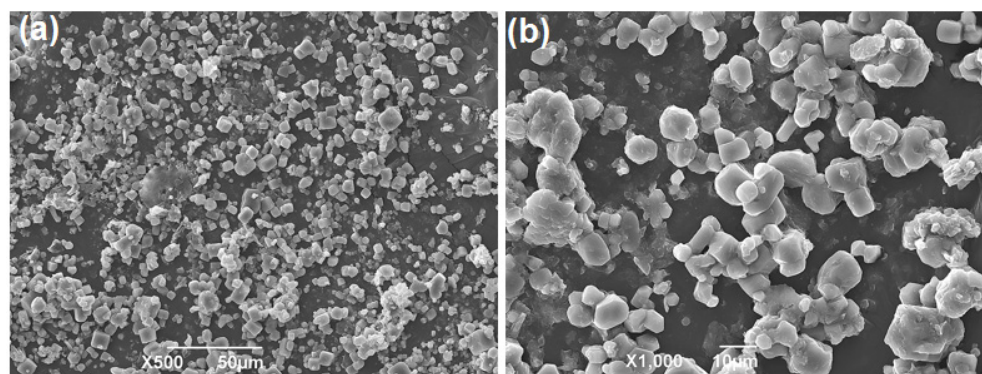


Figure 9. SEM micrograph of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source: (a) distribution of micro- and nano-pellets and (b) magnification of the obtained pellets, showing an irregular surface.

3.4.5. TGA Results

The thermal stability of PHB was analyzed by TGA, as shown in Figure 10. It can be seen from the TGA curve that PHB exhibited the first one-step thermal degradation with an onset at 85.6 °C and a minimum centered at 104.5 °C, while the second and most important process in the one-step thermal degradation was onset at 173.2 °C and its minimum was centered at 220.5 °C. Finally, the third change in the thermogram begins one-step thermal degradation with an onset at 407.5 °C and its minimum was centered at 468.7 °C. Additionally, Figure 10 depicts the TGA and the derivate weight profiles of PHB produced by *C. necator* using dextrose from cassava starch as a carbon source. The TGA curve shows the weight loss of the synthesized PHB in three stages. The first or initial stage of mass loss occurred in the temperature range of 85.6 °C to 173.2 °C. This mass loss can be associated with the loss of ambient humidity acquired by the hydrophilic characteristics of the PHB polymer. Moreover, this mass loss was approximately 1.0% of the total mass, which occurs due to the evaporation of physically adsorbed solvents such as sodium hypochlorite and chloroform on the polymer. Furthermore, the second and most relevant step in the degradation of the PHB was observed in the temperature range of 173.2 °C to 220.5 °C and can be related to a reduction in molecular weight, which includes chain scission and hydrolysis. The faster thermal degradation of PHB that occurs in this stage is obtained via the destruction of H-C-H from CH₃ groups, C=O ester carbonyl group, and alkyl (CH₃) halides, such as was observed in the FTIR results (Figure 7), and the destruction of crystalline regions associated with orthorhombic unit cells, such as was observed in the XRD results (Figure 6). The third step of weight loss was observed in the temperature range of 407.5 °C to 468.7 °C and occurs with a further rise in temperature, as hydrolysis also contributes to degradation, along with chain scission, with the subsequent formation of crotonic acid. Finally, it can be observed that the maximum degradation temperature for PHB obtained by *C. necator* is 451.8 °C. Comparing our results with other reports in the literature, a decomposition temperature 49 and 33% higher than the PHB obtained in other studies can be observed [56,57]. Therefore, the degradation temperature for the current polymer showed high thermal stability compared with other biopolymers [47]; this will allow it to be used in applications where high temperatures are required.

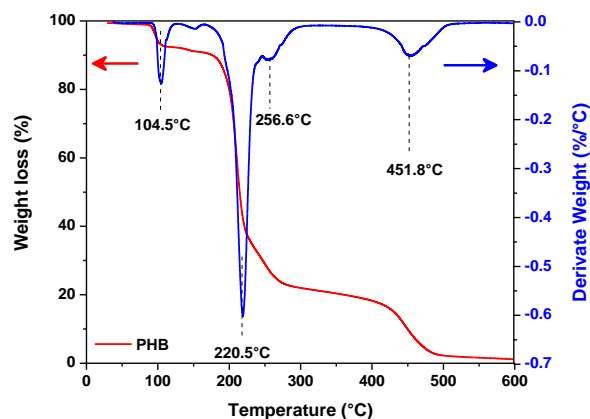


Figure 10. TGA of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source.

3.4.6. DSC Analysis

A DSC analysis was carried out to study the thermal nature of the PHB produced in this study (Figure 11); the second heating scan shows the melting temperature (T_m) of the polymer to be 173.4 °C. This value is similar to the standard PHB (Sigma Aldrich, St. Louis, MI, USA), which has a (T_m) of 177.0 °C, and to the reports in other studies for PHB (172.6 °C) [58]. From DSC analysis, the crystallinity degree (X_C) of PHB was calculated by comparing the area of the melting peak (ΔH_m) with the melting enthalpy of 100% crystalline PHB. From the technical references, the melting enthalpy of 100% crystal PHB is 146 Jg⁻¹ [59]. By using the reference value of the melting enthalpy together with the value of the melting enthalpy obtained for this biopolymer (55.6 Jg⁻¹), it can be observed that the degree of crystallinity for PHB produced in this study was 38%. Considering the last analysis of the X_C , the thermal degradation temperature, which can be associated with differences in structure, and the X_C observed from XRD results, the glass transition temperature (T_g) obtained for PHB ranges from −5 °C to 7 °C. So, according to the crystallographic analysis using XRD (Figure 6), the vibration modes of the bonds observed by FTIR spectroscopy (Figure 7), the compositional and morphological nature observed from the SEM-EDS results (Figures 8 and 9), and the thermal stability observed from TGA (Figure 10), the random, relatively crystalline nature of PHB hinders the initial movement of the polymer chain, resulting in the current glass transition temperature (−5 °C to 7 °C), melting enthalpy (55.6 Jg⁻¹), and X_C (38%).

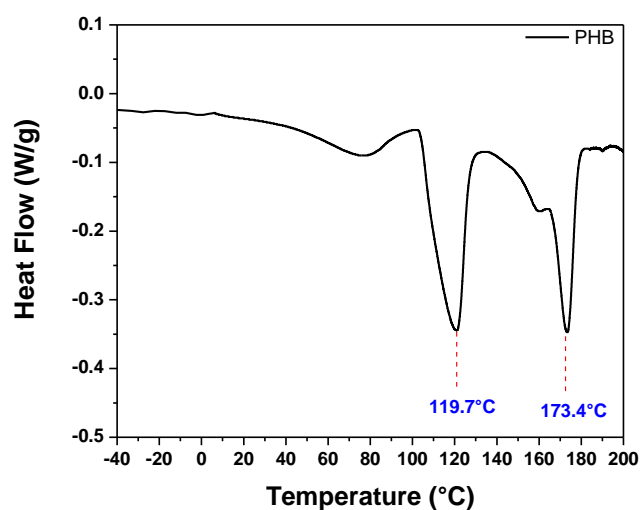


Figure 11. DSC characterization of PHB produced in submerged cultures of *C. necator* using dextrose from cassava starch as a carbon source (curve for heating cycle).

4. Conclusions

The current work presents a potential way to produce PHB via submerged cultures of *C. necator* using (i) only dextrose from Colombia cassava starch as a substrate and (ii) a combined substrate of dextrose and vinasse from the sugarcane industry thanks to their contribution as carbon sources and micronutrients, which makes them suitable low-cost carbon sources for obtaining the biopolymer. The most suitable culture medium for the growth of the bacteria and their subsequent production of PHB was found to be the one in which 7.5% of treated vinasse was used, reaching 2.98 ± 0.12 g/L of PHB (51% of accumulation), since the treatment of the vinasse has a positive impact on the growth of the microorganism by reducing the inhibitory agents present in the sugarcane vinasse, such as polyphenolic compounds. However, concerning the treatment of sugarcane vinasse, it is recommended to modify variables such as pH, temperature, and the amount of GAC, which influence the adsorption mechanism, to increase the removal of polyphenolic compounds present in the vinasse. In addition, cultures using only dextrose as a carbon source were also suitable for PHB production with 47.1% accumulation.

The PHB was characterized by XRD, FTIR, SEM-EDS, TGA, and DSC. The experimental results showed the crystallographic characteristics, the vibrational modes of the bonds, their compositional nature, their morphology, and their thermal stability. The PHB that was obtained had a glass transition temperature range of (-5 °C to 7 °C), a melting enthalpy of 55.6 Jg $^{-1}$, and a degree of crystallinity (XC) of 38%.

This approach is undoubtedly a potential valorization of industrial cassava starch and sugar industry vinasse to produce biodegradable bioplastics such as PHA.

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