Evaluation of Fed-Batch Fermentation for Production of Polyhydroxybutyrate With a Banana Pulp Juice Substrate From an Agro Industrial By-Product

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Pollution resulting from the persistence of plastics in the environment has driven the development of substitutes for these materials through fermentation processes using agro-industrial wastes. Polyhydroxybutyrate (PHB) is a rapidly biodegradable material with chemical and mechanical properties comparable to those of some petroleum-derived plastics. PHB accumulates intracellularly as an energy reserve in a wide variety of microorganisms exposed to nutritionally imbalanced media. The objective of this study was to evaluate the use of a banana waste product as a carbon source for PHB production. PHB was extracted by acid methanolysis and detected by gas chromatography-mass spectrometry. Eleven bacterial strains with potential for PHB production were evaluated by in vitro fermentation in a culture broth containing fructose as the carbon source and limited nitrogen. A $2^2$ central composite rotational design was applied to optimize the concentrations of banana juice and ammonium chloride needed to maximize the PHB-producing biomass concentration. The process was then carried out in a 3 L fed-batch fermentation system that included an initial stage of biomass growth. Banana juice was used as the carbon source and fructose pulses were added to maintain the test sugar concentrations of 30, 40, and 50 g/L. The control strain, Cupriavidus necator (ATCC 17699), produced 2.816 g/L of PHB, while productivity of the most promising isolate, C. necator (CR-12), was 0.495 g/L. Maximum biomass production was obtained using 5% banana juice and 2 g/L ammonium chloride. PHB production was not detected in fed-batch fermentations supplemented with 30 or 40 g/L of fructose, while the mean PHB production in fermentations with 50 g/L of fructose was 1.3 g/L.

Keywords: polyhydroxybutyrate, Cupriavidus necator, banana by-product, agro-industrial wastes, fed-batch fermentation, methanolysis
INTRODUCTION

Biotechnological alternatives to conventional plastics are increasingly important in industry worldwide (Sirohi et al., 2020) as they promote sustainability in the agriculture industry and strengthen rural economies (Li and Wilkins, 2021). Some types of biopolymers have valuable characteristics; they are biocompatible and biodegradable by enzymes, microorganisms and fungi present in the environment (Saralade et al., 2019; Sirohi et al., 2020) and can show thermoplastic characteristics with mechanical properties similar to materials derived from petroleum (Naranjo et al., 2014). Unlike biopolymers, petroleum-based products degrade slowly and take 20–100 years to decompose, which results in serious damage to the environment (Mozejko-Ciesielska and Kiewisz, 2016; Amaro et al., 2019; Sirohi et al., 2020). Therefore, there are strong incentives to develop alternative biodegradable materials that decompose rapidly to produce carbon dioxide, water and biomass (Kumar and Kim, 2018; Sirohi et al., 2020).

Polyhydroxybutyrate (PHB), the most common biodegradable homopolymer of polyhydroxyalkanoates (PHA) (Maity et al., 2020), has numerous applications in food, agriculture, medicine and other industries (Li et al., 2017; Sirohi et al., 2020). It naturally accumulates intracellularly in a large number of microorganisms (Oliveira et al., 2004; Mohapatra et al., 2020; Mostafa et al., 2020; Patti et al., 2020; Penkrhu et al., 2020; Nygaard et al., 2021). Some bacteria synthesize PHB as part of the primary metabolism; others, such as Cupriavidus necator, require a nutritional imbalance in the culture media to activate the synthesis metabolism (Grousseau et al., 2014; Getachew and Woldeyen, 2016; Amini et al., 2020).

The major limitation for commercial use of biopolymers is the high cost of production (Li et al., 2017; Li and Wilkins, 2021). For this reason, several studies have focused on the use of low-cost raw materials from agricultural residues (Sirohi et al., 2020) and fermentation methods that favor competitive production (Shivakumar, 2012; Sallau et al., 2018; Sukruansuwan and Naphathorn, 2018; Dalsasso et al., 2019), such as fed batch fermentation processes, which increase the productivity of PHB (Xia et al., 2017; Li and Wilkins, 2020, 2021; Nygaard et al., 2021).

In 2019, Costa Rica exported 120 million boxes of bananas (18.14 kg/box), which reached markets such as the European Union and the United States. The banana industry estimated 328,303 tons of fruit are rejected annually in packing plants (CORBANA, 2019). Rejected fruit has a high content of sugars such as glucose, sucrose and fructose, as well as a variety of minerals, vitamins and amino acids (Chan-Blanco et al., 2003; Guylène et al., 2009) that can be used in fermentative processes.

The objective of this research was to use banana pulp juice, an agroindustrial by-product as the carbon source for PHB production in a submerged fed-batch fermentation.

MATERIALS AND METHODS

Banana By-Product

The banana by-product, obtained from a pulp production process, was supplied by Compañía Mundimar (Limón, Costa Rica) and consisted of a puree with a high seed content. The material was subjected to enzymatic maceration with Crystalzyme® PMLX (White Labs) to increase the content of fermentable sugars. A pneumatic press was then used to obtain a juice from the banana puree (IBP) with a final concentration of 20% Brix and pH 3.6. Sugar content in IBP was measured by HPLC (Agilent 1260 Infinity). The instrument was equipped with a refractive index detector (RID) (Sullivan and Carpenter, 1993). Mineral content of the substrate was determined by atomic absorption spectrophotometry (AOAC International, 2012b,c,d,e,f). Nitrogen concentration was measured according to AOAC International (2012a).

Bacterial Strains

Eleven native isolates were provided by the Center for Research in Cellular and Molecular Biology of the Universidad de Costa Rica (CIBCM-UCR). These strains are part of a collection generated from samplings in different places in Costa Rica and have been obtained for various research projects of the CIBCM. The strains used in this study were selected from the positive reaction to Nile blue staining, a lipophilic fluorescent dye used to visualize hydrophobic cell structures such as membranes or lipid-like inclusions, like PHB (Juengert et al., 2018). A strain of Cupriavidus necator (ATCC 17699) was used as a control microorganism. The isolates were grown on Trypticase Soy Agar (TSA) (OXOID, CM0131, USA) at 30°C for 24 h. Cultures grown on TSA were used to prepare suspensions in Trypticase Soy Broth (TSB) (OXOID, CM0876, USA) to a concentration equivalent to standard 4 on the McFarland turbidity scale. The suspensions were frozen in 800 µL portions in cryovials with 200 µL 30% sterile glycerol until later use (Tedeschi and De Paoli, 2011).

PHB Extraction

Two mL of cultures were centrifuged at 10,600 rcf for 10 min. The cell pellets were resuspended in 2 mL of distilled water and transferred to 10 mL headspace vials. A volume of 6 mL of an 85:15 mixture of methanol:sulfuric acid (Dalal et al., 2010) was added to each sample. Sealed vials were incubated at 90–95°C for 3 h in a thermostatic bath with agitation for the methanolysis process (Cavalheiro et al., 2009; Dobroth et al., 2011; Wu et al., 2012; Davis et al., 2013). The samples were cooled in an ice bath and 1.5 mL of distilled water and 3 mL of chloroform were added. Samples were shaken vigorously and allowed to settle for phase separation. The organic phase was extracted (the sample was dried over anhydrous sodium sulfate) (Coats et al., 2008), and then filtered through a 0.22 µm filter and transferred to vials for Gas Chromatography (GC).

Quantification of PHB

Methyl (R)-3 hydroxybutyrate (3-MHB) (Sigma®, CAS: 3976-69-0) was used as a standard for PHB quantification (Cavalheiro et al., 2009) while benzoic acid was used as an internal standard (Merk, CAS: 65-85-0) (Coats et al., 2008). The methyl esters of 3-hydroxyalcanoic acid were analyzed by gas chromatography (7820A, Agilent Technologies) coupled to mass detection (5977B MSD Agilent Technologies). Detector was operated on Scan mode, with 70 eV ionization voltage. Two microliters samples
were injected into a HP-Innowax column, with a length of 30 m, internal diameter of 0.25 mm and width of 0.25 μm (Çiggin et al., 2009; Davis et al., 2013). The helium flow was 8 mL/min. A 20:1 split relation was used. The temperature of the injector and the detector was 250°C. Temperature was increased from 120 to 220°C at a rate of 3°C/min (Davis et al., 2013).

**Evaluation of PHB Production Capacity of Native Strains in Flask Culture**

Fermentations were carried out in two stages: a growth stage and a PHB production stage with limited nitrogen (Amirul et al., 2008; El-Sayed et al., 2009; Yang et al., 2010; Garcia-Gonzalez et al., 2015). For the first stage, 1 mL of cryopreserved culture was inoculated to 50 mL of TSB in 250 mL Erlenmeyer flasks. Cultures were incubated for 24 h at 30°C and 150 rpm in an orbital shaker (Lab Companion, SI-600, Germany). For the second fermentative phase, cultures were centrifuged at 3,320 rcf for 15 min in order to remove the growth medium, and the resulting cell pellet was resuspended in 50 mL of imbalanced culture media for PHB production described by Khanna and Srivastava (2005) with modifications: Fructose: 40 g/L, NH₄Cl: 5 g/L, KH₂PO₄: 1.5 g/L, NaH₂PO₄: 4 g/L, MgSO₄ * 7H₂O: 0.51 g/L, CaCl₂: 0.02 g/L, trace solution: 1 mL/L (trace solution composition [ZnSO₄ * 7H₂O: 13 mg/L, FeSO₄ * 7 H₂O: 2 mg/L, (NH₄)₂Mo₇O₂₄ * 4H₂O: 6 mg/L, H₂BO₃: 6 mg/L], Cultures were incubated at 30°C in an orbital shaker at 150 rpm for 48 h. Each strain was evaluated in triplicate. A Completely Randomized Design (CRD) was used with 12 treatments (11 native isolates and the control). The response variable was PHB concentration determined by GC-MS. Results were analyzed by one factor ANOVA using Microsoft Excel (version 18. 2008. 12711. 0).

**Design of Medium Supplemented With JBP for Growth of C. necator**

Nitrogen (NH₄Cl) and carbon (JBP) concentrations in culture media for C. necator (ATCC 17699) were optimized in order to maximize biomass growth. A 2² Central Composite Rotational Design was used to determine test concentrations of both components, according to Table 1. Four replicates of the central point were executed for statistical parameters calculation. Cell concentration was determined by measuring each culture optical density by spectrophotometry at 425 nm. A Response Surface Analysis was performed using JMP 8 software (SAS Institute Inc.).

For each experiment, 50 mL of culture broth supplemented with the test concentrations of JBP and NH₄Cl were prepared in 250 mL erlenmeyer flasks. Besides the test concentrations of NH₄Cl and JBP, the media contained 5 g/L NaCl and 2.5 g/L KH₂PO₄. For each broth formulation, JB, salts solution and water were combined. pH was adjusted to 7.1 with 1 M NaOH. NH₄Cl solution was autoclaved separately and aseptically added to each flask.

Each flask was inoculated with 1 mL of cryopreserved bacterial suspension and incubated at 30°C with 150 rpm agitation for 24 h. The optical density of the culture was measured at the end of the incubation.

### Table 1: JBP percentages and NH₄Cl concentrations evaluated by a central composite design for C. necator growth optimization

| Experiment | Design points | % juice | NH₄Cl (g/L) |
|------------|---------------|---------|-------------|
| 1          | 0             | 12.5    | 3.5         |
| 2          | ←             | 5       | 5           |
| 3          | A0            | 23      | 3.5         |
| 4          | 0a            | 12.5    | 1.4         |
| 5          | —             | 5       | 2           |
| 6          | a0            | 1.9     | 3.5         |
| 7          | 0A            | 12.5    | 5.6         |
| 8          | ←              | 20      | 2           |
| 9          | 0              | 12.5    | 3.5         |
| 10         | ++             | 20      | 5           |
| 11         | 0              | 12.5    | 3.5         |
| 12         | 0              | 12.5    | 3.5         |

**PHB Production Process Implementation in Bioreactor**

A fed-batch fermentation process was performed in triplicate using the C. necator strain (ATCC 17699). The process was carried out in stirred tank reactors (Applikon® Biotechnology) in a 3 L volume of culture media with the following composition: JBP: 5%; NaCl: 5 g/L; KH₂PO₄: 4 g/L, NH₄Cl: 2 g/L, Na₃HPO₄ * 2H₂O: 4.5 g/L, MgSO₄ * 7H₂O: 0.51 g/L, CaCl₂: 0.02 g/L and 10X trace element solution: 1 mL/L [ZnSO₄ * 7H₂O: 130 mg/L; FeSO₄ * 7 H₂O: 20 mg/L; (NH₄)₆Mo₇O₂₄ * 4H₂O: 60 mg/L; H₃BO₃: 60 mg/L]. The inoculum was prepared by adding 3 mL cryopreserved culture to 300 mL broth and incubating with shaking at 175 rpm for 24 h at 30°C (Aravind et al., 2015).

Fermentation parameters were controlled with the ez-Control console and BioXpert® XP software (Applikon® Biotechnology). Culture conditions were: temperature 30°C, agitation 350 rpm, pH 7 adjusted with 2 M NaOH and 100% air saturation at a flow of 1vvm. The biomass production stage was carried out over 24 h, and was followed by the PHB production stage, initiated by the addition of a fructose pulse (500 g/L), at an agitation speed of 500 rpm. In this stage, the effect of the initial concentration of fructose (30, 40 or 50 g/L) on PHB production was evaluated. Data were analyzed by one-way ANOVA using the program JMP 8 (SAS Institute Inc.). Samples were collected every 24 h; the concentration of PHB was determined by GC-MS, and sugar content was measured by HPLC (Agilent 1260 Infinity). The instrument was equipped with a refractive index detector (RID). A Zorbax Carbohydrate column with dimensions: 5 μm × 150 mm × 4.6 mm was used for the analysis. The following analytical conditions were applied: flow: 1.2 mL/min; oven and detector temperature; 30°C and injection volume: 5 μL. Nitrogen concentration was determined by Rapid N-Exceed® Combustion Method according to AOAC International (2016). The biomass dry weight concentration was determined in duplicate using a thermogravimetric analyzer (TGA 701, Leco).
RESULTS

Characterization of JBP
Enzymatic treatment of banana agroindustrial by-product produced a viscous concentrated juice. Chemical characterization showed values for fermentable sugars and minerals that can be used by the microorganism during fermentation. The ratio of sucrose, glucose and fructose in the JBP was 1.3:7.3:6, respectively. The nitrogen content was 0.056/100 g (Table 2).

PHB Production Capacity of Bacterial Strains
PHB production in native strains was not quantifiable (Measured PHB concentrations stood between detection and quantification limits defined for the method), except for CR-12 which showed a concentration of 0.5 g PHB/L culture broth. The control strain produced 2.82 g/L of culture broth. According to ANOVA, significant differences ($p < 0.05$) were found for PHB production means between the native strain CR-12 and control strain.

Design of JBP-supplemented Medium for Growth of C. necator
The effects of JBP ($p < 0.001$) and NH$_4$Cl ($p < 0.001$) concentrations, as well as both components interaction ($p = 0.0464$) was significant on C. necator cell growth, with a greater influence of JBP on optical density (Figure 1). The biomass concentration increased as levels of NH$_4$Cl and JBP decreased, as shown in Figure 2. Biomass concentration (OD) presented a linear behavior with an $R^2$ value of 0.99 (Table 3). Based on these results, concentrations of 2 g/L of NH$_4$Cl and 5% JBP were selected for use in fermentation experiments.

PHB Production Process Implementation in Bioreactor
Dissolved oxygen (DO) saturation was below 15% during the first stage of exponential growth, while levels of 20–40% were observed in the second stage of the process. Oxygen levels remained near zero during the first hours of fermentation. At the beginning of the fermentations, the fructose concentration was 2 g/L. After 25 h of culture, this value reached 50 g/L due to the fructose pulse added to initiate the PHB production stage. The sugar content then decreased and was completely depleted by the time the process reached 72 h. Little change in nitrogen concentration was observed at the end of the growth phase of the microorganism (Figure 3).

A biomass concentration of 24.42 ± 2.25 g/L was obtained at the end of the first fermentation stage. For fermentations where 30 and 40 g/L fructose were tested, PHB production was not detectable at any of the sampling times (24, 48, 72, and 96 h). A maximum PHB production of 1.3 g/L was reached after 96 h of fermentation for the 50 g/L fructose assay. In such experiments, C/N ratio was roughly 10, while C/N ratio obtained in fermentations with 30 and 40 g/L fructose was lower than 10.

In addition to methyl (S)-(+-)-3-hydroxybutyrate (PHB) signal, various fatty acids and carboxylic acids were also identified (Table 4).

DISCUSSION

Agro-industrial by-products can contain internal structures such as starches, cellulose and complex sugars that are difficult to transform directly by microorganisms into products of interest (Kulkarni et al., 2015; Sirohi et al., 2020). The enzymatic maceration pretreatment applied to the banana pulp allowed the conversion of the substrate into simple sugars molecules, primarily glucose (Velásquez-Arredondo et al., 2010) and fructose, as well as an increment on proteins and polysaccharides accessibility (Naranjo et al., 2014; Tsang et al., 2019), and facilitated the development of fermentation processes for PHB production. Even though the substrate enzymatic treatment increase the process costs, they are preferred over chemical treatments because the latter generate unwanted secondary
FIGURE 2 | Effect of different JBP and NH₄Cl supplementation levels in growth medium on C. necator cell concentration, measured as optical density at 425 nm.

TABLE 3 | Optical Density at 425 nm of C. necator in different formulations of biomass production culture media.

| Pattern | JBP (%) | NH₄Cl (g/L) | Optical Density 425 nm |
|---------|---------|-------------|-----------------------|
| +−      | 20      | 2           | 0.579                 |
| 0       | 12.5    | 3.5         | 1.731                 |
| −−      | 5       | 2           | 4.300                 |
| ++      | 20      | 5           | 0.254                 |
| −−      | 5       | 2           | 4.158                 |
| 0       | 12.5    | 3.5         | 2.078                 |
| −+      | 5       | 5           | 3.515                 |
| 0       | 12.5    | 3.5         | 1.896                 |
| +−      | 20      | 2           | 0.600                 |
| ++      | 20      | 5           | 0.273                 |
| 0       | 12.5    | 3.5         | 2.072                 |
| −−      | 5       | 2           | 3.983                 |
| −+      | 5       | 5           | 3.568                 |
| ++      | 20      | 5           | 0.304                 |
| −+      | 20      | 2           | 0.572                 |
| +−      | 5       | 5           | 3.403                 |
| 0       | 12.5    | 3.5         | 2.135                 |
| 0       | 12.5    | 3.5         | 2.196                 |

compounds (e.g., furfural) that negatively affect the development and metabolism of the microorganisms during fermentation (Lu and Zhong, 2020).

Regarding the native strains PHB production evaluation, the absence of this polymer in the majority such strains may be related to the individual nutritional requirements of the isolates. The optimization of production media is specific for the microorganism used (Pereira et al., 2021), this study used a base medium described for C. necator (suitable for control and CR-12 strains). The other strains may have been inhibited by components in the medium or may require absent nutrients from the utilized base medium for their metabolism proper functioning. PHB production with the native isolate CR-12 and the control strain was lower than that reported by other authors; Nygaard et al. (2019) obtained 4.6 g/L PHB using C. necator strain ATCC 17967 and Zhila et al. (2015) reported values of 7.2–7.7 g/L with strain B-10646 (C. eutrophus) in synthetic media in a two stage fermentation process. However, the PHB concentration of the control strain was higher than the maximum production of 1.62 g/L reported by Sen et al. (2019).

The concentrations of 2 g/L NH₄Cl and 5% JBP were optimum within the experimental range. However, results of the study indicate that higher biomass production may be obtained by
widen the experimental range to include lower concentrations of these two nutrients. For *C. necator*, some reports indicate that biomass production is maximized when glucose is used as the fermentation substrate, although other types of sugars such as fructose and xylose have also generated good results (Poomipuk et al., 2014). Given that the sugars in JBP are primarily fructose and glucose, this substrate was expected to maximize cell growth of the microorganism. The C/N ratio in the supplemented culture media was 5.3 based on the concentration and molar mass of glucose, fructose and \( \text{NH}_4\text{Cl} \). This value is below the range reported by Huschner et al. (2015); however, reducing the nitrogen content may result in a higher biomass concentration.

During fermentation processes implementation for PHB production, oxygen is an important parameter. *C. necator* biomass and PHB production have been observed to increase when the concentration of dissolved oxygen is maintained above 15% during the exponential growth phase of the microorganism in a fed-batch system (Cavalheiro et al., 2009). The effect of DO concentration on PHA production by *C. necator* has also been studied. Both the total concentration of PHAs and the proportion in which the polymers accumulate intracellularly were incremented when DO saturation was increased to 20% in the production phase, compared with the same process using a DO saturation of 2% (Cavalheiro et al., 2012). For all fructose treatments, the DO saturation increased in the second half of the process, and reached 20–40% for most of the consulted studies (Cavalheiro et al., 2009; Atlić et al., 2011; Passanha et al., 2013; Grousseau et al., 2014; Huschner et al., 2015; Dalsasso et al., 2019). In order to obtain PHAs, cultures with a high cell density are required. The biomass concentration in this study was 24.42 ± 2.25 g/L. Biomass values of 27 g/L have been obtained using complex carbon sources from agro-industrial by-products (Atlić et al., 2011; Cavalheiro et al., 2012).

In general, fructose is the most utilized carbon source for *C. necator*; a better PHB production is usually achieved by using this source than with glucose or other more complex sources such as...
as molasses (Baei et al., 2011). In preliminary experiments, PHB production was higher using this sugar as a carbon source (data not shown). Ienczak et al. (2013) obtained PHA yields of 68% (dry weight) when the biomass concentration was 50 g/L in a fedbatch system using glucose and fructose. For PHB production, the carbon source must be maintained at a high concentration with considerably limited nitrogen. In this research, maximum PHB production was 1.3 g/L at 96 h. This concentration is lower than that reported by other authors under similar conditions of oxygen saturation (Cavalheiro et al., 2009; Atlíć et al., 2011; Passanha et al., 2013; Grousseau et al., 2014; Huschner et al., 2015; Dalsasso et al., 2019), however, it is close to the concentration of 3.3 g/L reported by Li and Wilkins (2020) and 1.98 g/L obtained by Amini et al. (2020). A possible explanation for these results is the rapid consumption of fructose in the culture medium, which stimulates consumption of the polymer by the bacterial metabolism (Poomipuk et al., 2014). For fed-batch fermentations, PHB productivity was increased by replacing the single sugar supplement with several pulses of added sugar to maintain a relatively stable sugar concentration throughout the fermentation (Nygaard et al., 2021).

In this research, a roughly constant level of nitrogen was maintained; this may have prevented the C/N imbalance necessary for high PHB yields, or PHB synthesis may have been inhibited by the presence of nitrogen (Wang and Yu, 2007; Nygaard et al., 2021). The C/N ratio may explain the non-detectable levels of PHB in fermentations with fructose concentrations of 30 and 40 g/L. Huschner et al. (2015) found important percentages of PHAs using C/N ratios between 50 and 580, and obtained maximum productivity of C. necator ATCC 17699 at 48 h of culture using a C/N ratio of 90. Nygaard et al. (2019) considered the effect of phosphorous on PHB production and optimized culture media composition for C. necator ATCC 17967, maximum polymer production was 4.6 g/L when a C/N ratio of 10 was used.

Signals of other compounds have been detected during PHB analysis by GC-MS. Werker et al. (2008) suggested that these compounds were products of alcoholysis (or in the case of the current study, butanolysis), or were by-products of the degradation of the cell biomass or microbial metabolites. These authors used the NIST database to tentatively identify the signals for levulinic acid and the succinic acid dibutyl ester, compounds derived from polysaccharides present in the bacterial biomass and from microbial metabolism, respectively. As shown in Table 4, these two compounds were found in the fermentations as methyl ester and dimethyl ester, respectively. It is possible that the levulinic acid detected in various chromatograms was derived from microbial metabolism of the simple sugar used, or from the degradation of the C. necator biomass; levulinic acid can be used as a precursor for the synthesis of biopolymers (Sirohi et al., 2020).

Biosynthesis of PHAs and fatty acids share the same metabolic route with acetyl-CoA and propionyl-CoA as common intermediates. Thus, both routes compete for precursors, especially in nutrient-limited conditions. The mechanisms to explain this competition have not yet been elucidated, and further research is needed to be able to direct the synthesis process toward the component of interest, obtain high PHA productivities and control precisely the composition of these polymers (Magdouli et al., 2015). Consequently, it is possible that the fatty acids detected by GC-MS correspond to products of this metabolic route whose synthesis was favored over that of PHAs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CV-C contributed to obtaining the funds for the study. CV-C and MA-R did the conception and design of the study. MA-R and VM-V executed all the bacterial culture experiments to produce PHB, both by shaker flask and bioreactor, they extracted and determined the concentration of PHB by GC. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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