



Fed-batch production and characterization of polyhydroxybutyrate by *Bacillus megaterium* LVN01 from residual glycerol

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Abstract

The operating conditions of polyhydroxybutyrate (PHB) production processes are among the factors that most influence yields. In this study, we evaluated PHB production synthesized by *Bacillus megaterium* LVN01. Batch and fed-batch cultures were used to produce PHB from residual glycerol. For this, dry cell weight (DCW) and PHB productivity were analyzed at a preliminary stage by central composite design using batch systems under different temperature, C/N ratio, and fermentation time conditions. The maximum PHB productivity occurred at 30.8 °C, 44.9 mol mol⁻¹, and 39.9 h. The same conditions were tested for studies in fed-batch culture. Fed-batch experiments were comparable to each other, where the DCW was around 1.9 g L⁻¹, with PHB productivities of 29.5 mg L⁻¹ h⁻¹ and 35.6 mg L⁻¹ h⁻¹ for bioreactors of 5 L and 14 L, respectively. The PHB was characterized by NMR, FTIR, DSC, TGA, and DTG analyses.

Keywords: polyhydroxyalkanoates (PHA); biodiesel by-product; biopolymers extraction; PHB characterization; statistical experimental design.

Producción por lote alimentado y caracterización de polihidroxibutirato por *Bacillus megaterium* LVN01 a partir de glicerol residual

Resumen

Las condiciones de operación de los procesos de polihidroxibutirato (PHB) se encuentran entre los factores que más influyen en los rendimientos. En este estudio se evaluó la producción de PHB sintetizado por *Bacillus megaterium* LVN01. Se implementaron cultivos en lotes y lotes alimentados para producir PHB, a partir de glicerol residual. Para esto, en una etapa preliminar, se analizó el peso de las células secas (PSC) y la productividad de PHB mediante un diseño central compuesto, utilizando sistemas de lotes bajo diferentes condiciones de temperatura, relación molar C/N y tiempo de incubación. La productividad máxima de PHB ocurrió a 30.8°C, 44.9 mol mol⁻¹ y 39.9 h. Las mismas condiciones se utilizaron para estudios en cultivos por lotes alimentados. Los experimentos en modo lote alimentado fueron comparables entre sí, donde el PSC osciló alrededor de 1.9 g L⁻¹, con productividades de PHB de 29.5 mg L⁻¹ h⁻¹ y 35.6 mg L⁻¹ h⁻¹ para biorreactores de 5 L y 14 L, respectivamente. El PHB se caracterizó mediante análisis de RMN, FTIR, DSC, TGA y DTG.

Palabras clave: polihidroxialcanoatos (PHA); subproducto de biodiesel; extracción de biopolímeros; caracterización de PHB; diseño experimental estadístico.

1. Introduction

Worldwide polyhydroxyalkanoate (PHA) production continues to increase. In 2018, PHA production was about

29,000 tons, while it is expected to reach 37,000 tons by 2023 [1]. Microbial PHA are obtained through different strategies such as bioprocess (batch, fed-batch, and continuous) and by using cultures (pure and mixed) [2,3]. These strategies seek

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to increase yields in biomass and PHA production while reducing costs associated with the process. In this sense, economical substrates available from different industrial sectors are common alternatives for reducing the costs of PHA production.

PHAs have been synthesized by bacteria from a wide range of substrates, namely renewable sources (*e.g.*, sucrose, cellulose, hemicellulose, starch, and triglycerides), industrial by-products (*e.g.*, molasses, whey, and residual glycerol), organic acids (*e.g.*, 4-hydroxybutyric acid and propionic acid), fossil resources (*e.g.*, methane, mineral oil, and lignin), and water treatment residuals (*e.g.*, residual water and activated sludge effluent) [4]. The physics and chemical characteristics of PHAs synthesized by bacteria depend on the carbon source, the enzymatic machinery present in the strain, and the fermentation strategies used in the biopolymer production process [5,6].

Pure glucose is usually used as a substrate in PHA production as this is the natural substrate for PHA-producing microorganisms. However, this is an expensive substrate. Nonetheless, some studies have reported the use of low-cost industrial by-products. In this sense, residual glycerol, obtained as a by-product in the biodiesel industry, has become an accessible and readily available carbon source for chemical and microbiological transformations, such as PHA production by microorganisms [7,8].

On the other hand, commercial PHA production is mainly focused on the use of Gram-negative bacteria (*e.g.*, *Cupriavidus*, *Pseudomonas*, and *E. coli*) [9]. Nonetheless, these bacteria have endotoxins (lipopolysaccharides, LPS), which are released in the biopolymer extraction processes and affect their subsequent application in some areas of the industry (*e.g.*, medical and pharmaceutical), thus requiring additional stages of purification [10]. In contrast, Gram-positive bacteria, specifically those belonging to the genus *Bacillus*, have been widely studied as PHA producers from different agro-industrial residuals, increasing interest in these at the industrial level [11]. One of their advantages is the lack of LPS, which makes purification stages after the production process of these biopolymers unnecessary. The absence of these steps reduces production costs, which is one of the main challenges facing the PHA industry's petroleum-derived plastics.

About 150 different monomers have been identified within this type of biopolymer [12]. The chemical structure of each type of PHA, and therefore its industrial application, depends on the type of microorganism, its enzymatic capabilities, and the carbon sources [13]. Consequently, the characterization of PHA synthesized by microorganisms is one of the fundamental parts of the production process.

In this study, polyhydroxybutyrate (PHB) production by *B. megaterium* LVN01 was evaluated under different operating conditions (C/N ratio, temperature, and incubation time) and different operation modes (batch and fed-batch). Residual glycerol obtained as a by-product of biodiesel production was implemented as the substrate. The PHB characterization was performed by Nuclear Magnetic Resonance (NMR) Spectroscopy and Fourier Transform

Infrared Spectrometry (FTIR), and some physical properties were determined by Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), and Differential Thermogravimetric Analysis (DTG).

2. Methods

2.1. Bacterial strain and inoculum preparation

All experiments were performed with *B. megaterium* LVN01 (GenBank: QJGY00000000.1), which was isolated from soil samples in Guarne (06°16'50"N, and 75°26'37"W, Antioquia, Colombia) [14]. Lysogeny broth (LB - 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) was used in this study, and it was supplemented with 1.4% agar as a solid medium. Cells were cultivated on LB agar plates at 37 °C for 24 h. One colony of *B. megaterium* LVN01 was used to inoculate 10 mL of LB. The culture flasks were placed on a rotary shaker and incubated overnight at 37 °C and 200 rpm. After incubation, 1 mL of culture broth was inoculated in 100 mL Erlenmeyer flasks containing 9 mL of fresh LB and incubated on a rotatory shaker at 37 °C and 200 rpm for 2 h (or until the optical density at 600 nm (OD₆₀₀) was 0.5 - 0.6). This culture broth was used as inoculum for the following steps.

2.2. Carbon source

All experiments were performed using residual glycerol kindly provided by a Colombian biodiesel company (Bio D S.A Company). Donated samples corresponded to batch number GC00555TK452 and contained 83.1% (w/w) glycerol (Table S1).

2.3. Batch Process. Effect of the C/N ratio, temperature, and fermentation time on DWC and PHB productivity

Batch cultures were carried out using residual glycerol synthetic medium (WGM) that contained, 1.0 g L⁻¹ yeast extract, 1.5 g L⁻¹ KH₂PO₄, 3.6 g L⁻¹ Na₂HPO₄, 0.2 g L⁻¹ MgSO₄·7H₂O, and 1 mL of trace element solution (TES). TES contained: 10.0 g L⁻¹ FeSO₄·7H₂O, 2.25 g L⁻¹ ZnSO₄·7H₂O, 1.0 g L⁻¹ CuSO₄·5H₂O, 0.5 g L⁻¹ MgSO₄·5H₂O, 2.0 g L⁻¹ CaCl₂·2H₂O, 0.23 g L⁻¹ Na₂B₄O₇·10H₂O, 0.1 g L⁻¹ (NH₄)₆Mo₇O₂₄, and 10 mL L⁻¹ of HCl 35%, supplemented with residual glycerol as a carbon source and ammonium sulfate as a nitrogen source at different molar ratios (Table 1). An inoculum of 3 mL of *B. megaterium* LVN01 was added to 300 mL of WGM in a 1 L Erlenmeyer flask, which was incubated for 8 h at different temperatures, according to the test conditions. Finally, this pre-culture was used to inoculate 2.7 L of WGM culture broth at OD₆₀₀ of 0.1. *B. megaterium* LVN01 was incubated in a 5 L bioreactor (Centricol, stirred tank series 01240) at 400 rpm, with aeration controlled by extra dry oxygen pulses to maintain aerobic conditions and with an initial pH value of 7.0. At the beginning of each batch, drops of 10% (v/v) antifoam solution (silicone oil) were added to the medium.

Table 1.

Operational conditions to determine the effect of the C/N ratio, temperature, and fermentation time on DCW and PHB productivity

Factors	Values		
C/N ratio (mol mol ⁻¹)	21.6	33.2	44.9
Time (h)	36	48	72
Temperature (°C)	30	32.5	35

C: residual glycerol (20, 25 y 30 g L⁻¹); N: (NH₄)₂SO₄ (0.8, 1.4 y 2.5 g L⁻¹)
Source: The Authors.

Growth was monitored spectrophotometrically by estimating the OD₆₀₀ in a spectrophotometer (Shimadzu UV-1800) and with the determination of dry weight cell (DWC).

To determine the effect of the variables (C/N ratio, temperature, and incubation time) on DWC and PHB productivity, a central composite design was implemented and analyzed using response surface methodologies. The operating conditions for obtaining higher PHB yields by *B. megaterium* strains reported in the literature were considered as the starting point in this study.

2.4. Fed-batch process

Fed-batch experiments were performed in 2 bioreactors, one of 5 L (Centricol, stirred tank series 01240) with a working volume of 3 L, and another of 14 L (New Brunswick BioFlo® / CelliGen® 115) with a working volume of 6 L. The parameters obtained in the batch and fed-batch approaches using the 5 L bioreactor were implemented in the 14 L bioreactor in the final scale-up stage. The bioreactors were operated at a stirring speed of 400 rpm. The dissolved oxygen was set at 100% saturation, using extra-dry industrial oxygen at the beginning of the processes. The initial pH value was set at 7.0. Drops of 10% (v/v) antifoam solution (silicone oil) were added when necessary. Bacterial growth was monitored spectrophotometrically by estimating the OD₆₀₀ and by determining the DCW.

Initial working volumes for the 5 L and 14 L bioreactor were 2.5 L and 5.5 L, respectively. Residual glycerol (200 g L⁻¹) was added 12 h and 24 h after inoculation, until a total workload of 3 L and 6 L was completed for the 5 L and 14 L bioreactors, respectively. The microorganism was incubated over 84 h. Triplicate independent samples were taken at each stage of the process.

2.5. PHB quantification and productivity

PHB quantification was performed using the crotonic acid method [15], with some modifications. The PHB was isolated from cells by extraction with NaClO 15 % (v/v):CHCl₃ dispersion at 40 °C for 3 h in screw-cap tubes. Afterwards, the mixture was centrifuged at 8000 xg for 15 min at 20 °C, and the organic phase containing the PHB was filtered. An aliquot of 0.1 mL of the filtered solution was dissolved in 0.9 mL of concentrated sulfuric acid (H₂SO₄ 95-97%). The solution was heated at 100 °C for 10 min, and then the mixture was cooled on ice. Finally, absorbance at 235 nm was measured in a UV-VIS spectrophotometer. The calibration curve was constructed by preparing solutions of

known concentrations of crotonic acid in concentrated H₂SO₄ (Fig. S1). The solutions tested were compared against a concentrated H₂SO₄ blank.

PHB productivity was calculated as shown in eq. (1) and should be understood as the PHB concentration obtained using the methodology described above, which was produced in the WGM culture per unit of time. Triplicate independent samples were taken at each stage of the experiment.

$$PHB \text{ productivity} = \frac{PHB \text{ concentration (mg L}^{-1}\text{)}}{time (h)} \quad (1)$$

2.6. Ammonium quantification

Residual ammonium quantification was determined following the methodology described by Solórzano [16], which quantifies a type of dye (indophenol blue), a molecule obtained from the reaction between phenol, sodium hypochlorite and ammonium, and catalyzed by sodium nitroprusside, at 640 nm through spectrophotometry. The calibration curve was constructed using (NH₄)₂SO₄ solutions of known concentration.

2.7. PHB characterization

2.7.1. Fourier Transform Infrared spectroscopy (FTIR)

Material extracted from *B. megaterium* LVN01 cells and commercial PHB (Sigma-Aldrich, poly(3-hydroxybutyric acid)) were analyzed using FTIR. Spectra were recorded using a PerkinElmer Spectrum Two FTIR spectrometer. For each measurement, 32 scans were acquired and averaged in the range of 4000 cm⁻¹ to 400 cm⁻¹ with a spectral resolution of 4 cm⁻¹.

2.7.2. Nuclear Magnetic Resonance (NMR)

¹H and ¹³C NMR spectra of material obtained from *B. megaterium* LVN01 and commercial PHB (Sigma-Aldrich), were recorded using a Bruker UltraShield Fourier 300 NMR spectrometer at 300 MHz, using tetramethylsilane as the internal standard. For each measurement, between 5 and 15 mg of biopolymer was dissolved in 0.5 mL of CDCl₃.

2.7.3. Differential Scanning Calorimetry (DSC)

Melting temperature (*T_m*) analyses were performed on a Mettler Toledo DSC 1 STARe System Calorimeter. Approximately 6 mg of the material extracted from *B. megaterium* LVN01 cells was subjected to a heating curve from 25°C to 200°C, with a heating ramp of 10°C min⁻¹, under an inert nitrogen atmosphere.

2.7.4. Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTG)

Decomposition temperature (*T_d*) was determined in a NETZSCH Thermogravimetric Analyzer TG 209 F1 Libra. Samples were processed at a speed of 10 °C min⁻¹ from 25 °C to 600 °C, under an inert nitrogen atmosphere. For this

study, 2.5 mg of the extracted material was taken for each test. A DTG plot was obtained by calculating the first derivative of the data obtained for TGA.

3. Results and discussion

3.1. Batch process

A preliminary approach used shake flasks in the process, in order to understand the microorganism's behavior [17]. This section shows the results obtained for the scale-up of the *B. megaterium* LVN01: residual glycerol system in a 5 L bioreactor. Initially, the performance of the microorganism-substrate system was evaluated in a 5 L bioreactor with a working volume of 3 L under different C/N ratio, temperature, and incubation time conditions. The configuration of the independent variables for different assays was established by a central composite design. The expected values of DCW and PHB production, which is expressed in terms of PHB productivity, were fitted by

complete second-order models, using the response surface methodology (Table 2).

The quadratic effect of incubation time on the DCW (Table 3) and incubation time on PHB productivity (Table 4) were found to be significant ($p < 0.05$). The fitted models explain 70% of the variability in DCW and 64% for PHB productivity. Likewise, the values that optimize the system for DCW (C/N ratio 21.6 mol mol⁻¹, 32.8 °C and 58.9 h) and PHB productivity (C/N ratio 44.9 mol mol⁻¹, 30.8 °C and 39.9 h) were established by analyzing the response surfaces (Fig. 1). These values are within the highest ranges reported for PHB production from glycerol by different strains of *B. megaterium* [18,19].

3.2. Fed-Batch process (5 L bioreactor)

Based on the above results, a 5 L bioreactor was used in fed-batch mode. Fig. 2 plots the values of the response variables obtained in the experiments carried out in a 5 L bioreactor for 84 h at 30.8 °C with a C/N ratio of 44.9 mol mol⁻¹.

Table 2. Evaluation of different batch culture operating conditions for the *B. megaterium* LVN01 system:residual glycerol.

Ratio C/N (mol mol ⁻¹)	Temperature (°C)	Time (h)	DWC (g L ⁻¹)	Productivity (mg PHB L ⁻¹ h ⁻¹)
33.2	32.5	48	0.50	7.6
44.9	30	36	0.70	13.8
21.6	35	36	0.48	7.9
44.9	32.5	48	0.92	11.2
21.6	35	72	1.26	8.8
44.9	35	36	0.51	10.3
33.2	30	48	0.62	7.3
21.6	35	72	0.76	3.2
33.2	32.5	72	0.50	1.8
44.9	30	72	0.49	5.4
21.6	30	72	1.03	6.7
33.2	35	48	1.00	12.8
33.2	32.5	48	0.59	8.9
33.2	32.5	36	0.48	12.3
21.6	30	36	0.50	10.8
21.6	32.5	48	2.10	15.5

Source: The Authors.

Table 3. ANOVA for DCW of the *B. megaterium* LVN01:residual glycerol system.

Source	Sum of squares	Df	Mean square	F-ratio	P-value
A: C/N ratio	0.2790	1	0.2790	1.97	0.2096
B: Temperature	0.0017	1	0.0017	0.01	0.9154
C: Time	0.0458	1	0.0458	0.32	0.5896
AA	0.6053	1	0.6053	4.28	0.0839
AB	0.0094	1	0.0094	0.07	0.8050
AC	0.2294	1	0.2294	1.62	0.2497
BB	0.0272	1	0.0272	0.19	0.6763
BC	0.0017	1	0.0017	0.01	0.9173
CC	0.8759	1	0.8759	6.20	0.0472
Total error	0.8479	6	0.1413		
Total (corr.)	2.8219	15			
Adj R ² = 70%					

Source: The Authors.

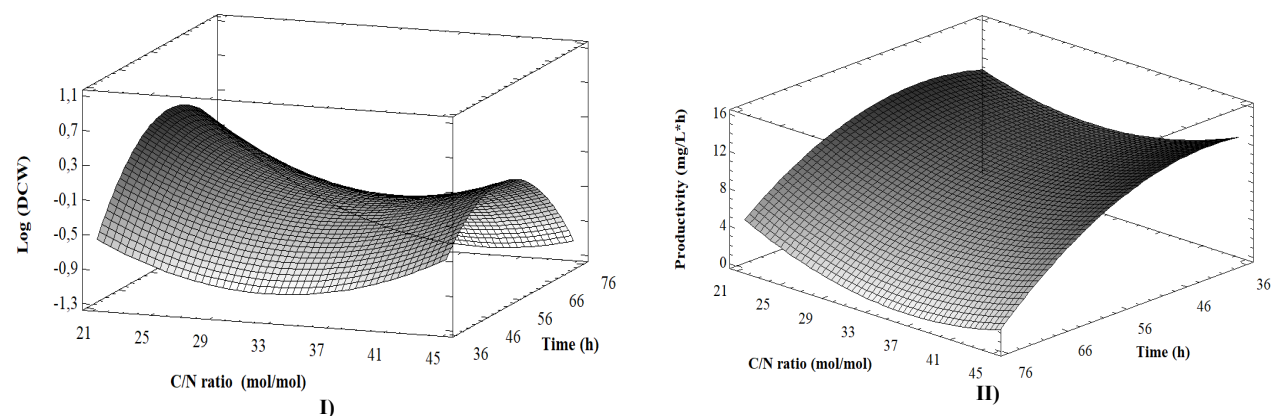


Figure 1. Fitted response surfaces for (I) DCW and (II) PHB productivity from the *B. megaterium* LVN01:residual glycerol system at 30 °C. Source: The Authors.

Table 4.
ANOVA for PHB productivity of the *B. megaterium* LVN01: residual glycerol system.

Source	Sum of squares	Df	Mean square	F-ratio	P-value
A: C/N ratio	1.3213	1	1.3213	0.11	0.7536
B: Temperature	1.3383	1	1.3383	0.11	0.7520
C: Time	73.716	1	73.716	6.03	0.0494
AA	14.742	1	14.742	1.21	0.3143
AB	0.2932	1	0.2932	0.02	0.8820
AC	5.4283	1	5.4283	0.44	0.5300
BB	0.9201	1	0.9201	0.08	0.7931
BC	0.0889	1	0.0889	0.01	0.9348
CC	16.127	1	16.127	1.32	0.2945
Total error	73.377	6	12.229		
Total (corr.)	206.00	15	1.3213		

Adj R² = 64%

Source: The Authors.

Results in the 5 L bioreactor show that the DCW in fed-batch mode increased compared to using the same reactor under the same operating conditions but in batch mode. As shown in Fig. 2, DCW and PHB productivity reached 16.6 mg L⁻¹ h⁻¹ and 1.96 g L⁻¹ respectively in the fed-batch system, after 48 h of incubation. However, the highest productivity was observed at 12 h (29.5 mg L⁻¹ h⁻¹) with a DCW of 0.97 g L⁻¹. This indicates that *B. megaterium* LVN01 accumulates PHB faster during the first 12 h of incubation. These results are slightly higher than those previously reported for *B. megaterium* MTCC10086, with DCW values of 1.54 g L⁻¹ and a PHB productivity of 13.5 mg L⁻¹ h⁻¹ [20].

Meanwhile, the concentration of ammonium throughout the experiment remained almost constant (Fig. S2). Since *B. megaterium* LVN01 requires yeast extract in the culture medium to be able to grow in the residual glycerol, it is evident that ammonium sulfate is not involved in its metabolism (Fig. S3). This strain displays better acceptance of yeast extract as the nitrogen source, instead of ammonium

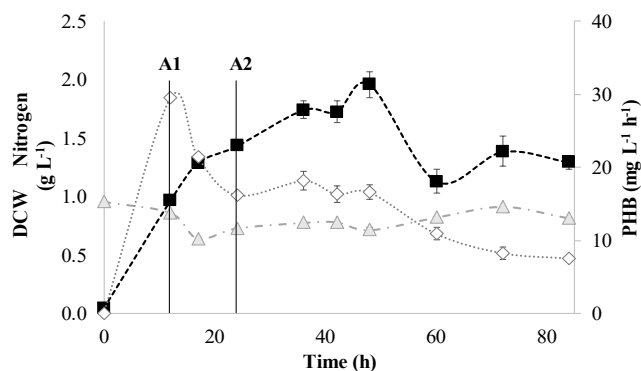


Figure 2. Fed-batch system in a 5 L bioreactor. (◇) PHB Productivity (mg L⁻¹ h⁻¹); (△) ammonia nitrogen (g L⁻¹); (■) DCW (g L⁻¹); A1 (12 h) and A2 (24 h) represent glycerol feed times.

Source: The Authors.

sulfate, which means that the concentration of ammonium sulfate remains constant. Similarly, it has been observed that yeast extract directly affects the growth of *B. megaterium* strains that use glycerol as a carbon source, whereas increased concentration of yeast extract in the medium leads to a decrease in both DCW and PHA productivity [19].

3.3. Fed-Batch process (14 L bioreactor)

Experiments in a 14 L bioreactor were performed with a working volume of 6 L. Fig. 3 illustrates the results obtained in the 14 L bioreactor operating in fed-batch mode for 84 h, under the same operating conditions used in the 5 L bioreactor (i.e., 30.8 °C and C/N ratio of 44.9 mol mol⁻¹).

As can be seen in Fig. 3, the trends in the data are similar to the data obtained in the 5 L bioreactor. However, it was observed that the highest DCW (1.91 g L⁻¹) occurs at 36 h, slightly higher than the 1.74 g L⁻¹ obtained in the 5 L bioreactor at the same point. This can be explained by the long stationary phase of the system under study. Thus, it can be seen that the system is in a stationary phase between 36 and 48 h for both study scales (5 L and 14 L) and that the system is at its maximum biomass production.

On the other hand, the higher productivity of PHB (35.6 mg L⁻¹ h⁻¹) is again evidenced at 12 h (slightly higher than the 29.5 mg L⁻¹ h⁻¹ obtained with a culture volume of 5 L). These results are similar to PHB productivities reported with *Bacillus* strains using glycerol as a carbon source [21,22].

Likewise, the same behavior with the nitrogen source was observed, which, as mentioned above, has also been reported elsewhere. This evidence confirms that yeast extract serves as the main nitrogen source for *B. megaterium* LVN01, and the acceptance of residual glycerol as a carbon source is possible only in the presence of yeast extract. It should be noted that other studies have also reported poor assimilation of ammonia nitrogen by strains of *B. megaterium* [23].

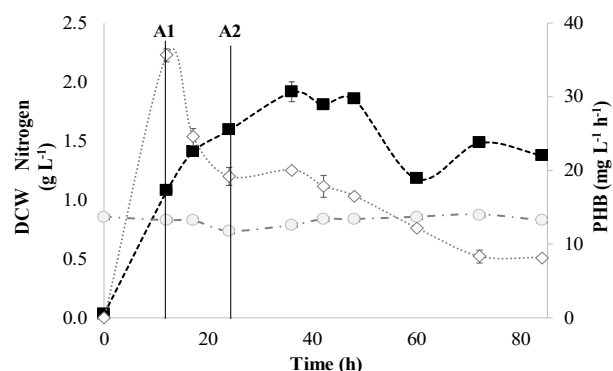


Figure 3. Fed-batch system in a 14 L bioreactor. (◇) PHB Productivity (mg L⁻¹ h⁻¹); (○) ammonia nitrogen (g L⁻¹); (■) DCW (g L⁻¹); A1 (12 h) and A2 (24 h) represent glycerol feed times.

Source: The Authors.

3.4. Functional groups identified by FTIR

The identification of the biopolymer's functional groups was carried out using FTIR. The bands in the spectrum at 2976 cm^{-1} and 2932 cm^{-1} correspond to the stretching of CH groups. The signal at 1720 cm^{-1} corresponds to the stretching of the carbonyl group (C=O). 1280 cm^{-1} corresponds to the stretching of the ester group (C-O). The bands at 1380 cm^{-1} , 1228 cm^{-1} , and 1180 cm^{-1} correspond to groups CH₃, CH₂, and C-O-C, respectively. Based on these results, it was determined that the spectrum corresponds to the characteristic signals of the PHB (Fig. S4) [24,25].

3.5. Chemical structure elucidation by NMR

The chemical structure of the biopolymer produced was elucidated by ¹H and ¹³C NMR. The signals at δ_{1H} : 1.30 ppm indicate the presence of the methyl group (CH₃), 2.50 - 2.64 ppm for the methylene group (CH₂), 5.28 ppm for the (CH) group (Fig. S5), and δ_{13C} : 169.20, 67.65, 40.81, and 19.80 ppm, which represent C=O, CH, CH₂ and CH₃, respectively (Fig. S6) [26,27].

The comparison of the NMR spectrum of commercial PHB with the spectrum of the PHA produced by *B. megaterium* LVN01 confirms that this microorganism produces PHB (Fig. S5 and S6).

3.6. Determination of the melting temperature by DSC

The melting temperature (T_m) and the melting enthalpy (ΔH_f) of the PHB synthesized by *B. megaterium* LVN01 from residual glycerol was analyzed by DSC (Fig. S7). Thus, the degree of crystallinity (X_{cr}) could be calculated using eq. (2).

$$X_{cr}(\%) = 100x \frac{\Delta H_f}{\Delta H_f^\circ} \quad (2)$$

Where:

ΔH_f : Enthalpy of fusion of PHB produced by *B. megaterium* LVN01

ΔH_f° : Enthalpy of fusion of pure PHB, equivalent to 146 J g⁻¹ [28].

For PHB, typical T_m values are 130-180 °C [26,28], and X_{cr} values are 30-60% [20,30]. In this study, the T_m was equal to 168.9 °C. The ΔH_f for the PHB was calculated as the area under the T_m curve. Finally, X_{cr} was calculated to be equal to 35.7%. These results correspond to the typical reported values for PHB synthesized by *B. megaterium* [18,31].

3.7. Thermal stability by TGA and DTG analyses

The PHB produced by *B. megaterium* LVN01 was analyzed through TGA and DTG to assess its thermal stability. The degradation of the PHB occurs in a simple process and starts around 230 °C, reaching its maximum decomposition temperature at 266.2 °C. The evaluation of the sample's weight loss indicates that between 230 and

300 °C, 97.7% of the mass was lost (Fig. S8). As was expected, the values obtained in this study are similar to those reported for PHB elsewhere [18,26,27].

4. Conclusions

In a preliminary approach, batch cultures were used to establish optimal growth conditions for *B. megaterium* LVN01: residual glycerol system. It was determined that the best operating conditions were 30.8 °C, 39.9 h of incubation and a C/N ratio of 44.9 mol mol⁻¹. In the case of the C/N ratio, the importance of the concentration of glycerol (substrate) instead of ammonium sulfate (nitrogen source) was established, as the ammonium remains almost constant throughout the processes.

The studies in 5 L and 14 L quantities established that 12 h of incubation displayed the highest PHB productivity (29.5 and 35.6 mg L⁻¹ h⁻¹, respectively). It was also determined that there is a stationary phase of at least 12 h when *B. megaterium* LVN01 presents the highest DCW, with values greater than 1.9 g L⁻¹ in both bioreactors operating in fed-batch mode.

On the other hand, the significant effect of the presence/absence of yeast extract on the growth of *B. megaterium* LVN01 in residual glycerol was determined, leading to the conclusion that without this supplement, this strain is not capable of assimilating residual glycerol as a carbon source (*data not shown*).

Finally, the physical and chemical characterizations of the PHA synthesized by the *B. megaterium* LVN01 strain from residual glycerol were performed. FTIR, ¹H-NMR, and ¹³C-NMR spectra allowed the chemical structure of the synthesized compound to be elucidated, and it was found that the monomer is 3-hydroxybutyrate (3HB). The biopolymer was therefore concluded to be PHB. The study of some physical and chemical properties of PHB established its melting (T_m) and decomposition temperatures (T_d), as well as its degree of crystallinity (X_{cr}). These values are within the reported ranges for PHB synthesized by strains of *B. megaterium*.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

- [1] European Bioplastics., Report-Bioplastics market data 2018, Nova Institute, 2018, 4 P.
- [2] Coats, E.R., Watson, B.S. and Brinkman, C.K., Polyhydroxyalkanoate synthesis by mixed microbial consortia cultured on fermented dairy manure: effect of aeration on process rates/yields and the associated microbial ecology. *Water Research*, 106, pp. 26-40, 2016. DOI: 10.1016/j.watres.2016.09.039
- [3] Rodríguez-Perez, S., Serrano, A., Panti6n, A.A. and Alonso-Fari6nas, B., Challenges of scaling-up PHA production from waste streams. A review. *Journal of Environmental Management*, 205, pp. 215-230, 2018. DOI: 10.1016/j.jenvman.2017.09.083
- [4] Urtuvia, V., Villegas, P., Gonz6lez, M. and Seeger, M., Bacterial production of the biodegradable plastics polyhydroxyalkanoates. *International Journal of Biological Macromolecules*, 70, pp. 208-213, 2014. DOI: 10.1016/j.ijbiomac.2014.06.001
- [5] Bugnicourt, E., Cinelli, P., Lazzeri, A. and Alvarez, V., Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging. *Express Polymer Letters*, 8(11), pp. 791-808, 2014. DOI: 10.3144/expresspolymlett.2014.82
- [6] Laycock, B., Arcos-Hernandez, M.V., Langford, A., Pratt, S., Werker, A., Halley, P.J. and Lant, P.A., Crystallisation and fractionation of selected polyhydroxyalkanoates produced from mixed cultures. *New Biotechnology*, 31(4), pp. 345-356, 2014. DOI: 10.1016/j.nbt.2013.05.005
- [7] Da Silva, G.P., Mack, M. and Contiero, J., Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnology Advances*, 27(1), pp. 30-39, 2009. DOI: 10.1016/j.biotechadv.2008.07.006
- [8] Zhu, C., Chiu, S., Nakas, J.P. and Nomura, C.T., Bioplastics from waste glycerol derived from biodiesel industry. *Journal of Applied Polymer Science*, 130(1), pp. 1-13, 2013. DOI: 10.1002/app.39157
- [9] Yatim, A., Syafiq, I., Huang, K., Amirul, A., Effendy, A. and Bhubalan, K., Bioconversion of novel and renewable agro-industry by-products into a biodegradable poly(3-hydroxybutyrate) by marine *Bacillus megaterium* UMTKB-1 strain. *BioTechnologia*, 98(2), pp. 141-151, 2017. DOI: 10.5114/bta.2017.68313
- [10] Park, S.J., Kang, K.H., Lee, H., Park, a.R., Yang, J.E., Oh, Y.H., Song B.K., Jegal, J., Lee, S.H. and Lee, S.Y., Propionyl-CoA dependent biosynthesis of 2-hydroxybutyrate containing polyhydroxyalkanoates in metabolically engineered *Escherichia coli*. *Journal of Biotechnology*, 165(2), pp. 93-98, 2013. DOI: 10.1016/j.jbiotec.2013.03.005
- [11] Kumar, P. Patel, S., Lee, J-K. and Kalia, V.C., Extending the limits of *Bacillus* for novel biotechnological applications. *Biotechnology Advances*, 31, pp. 1543-1561, 2013. DOI: 10.1016/j.biotechadv.2013.08.007
- [12] Yeo, J.C.C., Muiruri, J.K., Thitsartarn, W., Li, Z. and He, C., Recent advances in the development of biodegradable PHB-based toughening materials: approaches, advantages and applications. *Materials Science and Engineering C*, 92(2018), pp. 1092-1116, 2018. DOI: 10.1016/j.msec.2017.11.006
- [13] S6nchez, S.A., Mar6n, M.A., Mora, A.L. and Yepes, M.S., Identification of polyhydroxyalkanoate-producing bacteria in soils contaminated with fique wastes. *Revista Colombiana de Biotecnologia* [Online]. 14(2), pp. 89-100, 2012. [date of reference April 12th of 2019]. Available at: http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0123-34752012000200010
- [14] Law, J.H. and Slepecky, R., Assay of poly- β -hydroxybutyric acid. *Journal of Bacteriology* [Online]. 82(1), pp. 33-36, 1961. [date of reference April 12th of 2019]. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC279110/>
- [15] Sol6rzano, L., Determination of ammonia in natural seawaters by the phenol-hypochlorite method. *Limnology and Oceanography*, pp. 799-801, 1969. [date of reference April 12th of 2019]. Available at: <https://aslopubs.onlinelibrary.wiley.com/doi/abs/10.4319/lo.1969.14.5.0799>
- [16] Moreno, P., Ya6nez, C., Cardozo, N.S.M., Escalante, H., Combariza, M.Y. and Guzman, C., Influence of nutritional and physicochemical variables on PHB production from raw glycerol obtained from a Colombian biodiesel plant by a wild-type *Bacillus megaterium* strain. *New Biotechnology*, 32(6), pp. 682-689, 2015. DOI: 10.1016/j.nbt.2015.04.003
- [17] G6mez, J.R., Mora, A.L., Yepes, M.S. and Correa, G.A., Production and characterization of polyhydroxyalkanoates and native microorganism synthesized from fatty waste. *International Journal of Polymers Science*, 2016, pp. 1-12, 2016. DOI: 10.1155/2016/6541718
- [18] De Jesus Assis, D., Gomes, G.V.P., da Cunha Pascoal, D.R., Pinho, L.S., Chaves, L.B.O. and Druzian, J.I., Simultaneous biosynthesis of polyhydroxyalkanoates and extracellular polymeric substance (EPS) from crude glycerol from biodiesel production by different bacterial strains. *Applied Biochemistry and Biotechnology*, 180(6), pp. 1110-1127, 2016. DOI: 10.1007/s12010-016-2155-z
- [19] Porras, M.A., Vitale, C., Villar, M.A. and Cubitto, M.A., Bioconversion of glycerol to poly(HB-co-HV) copolymer in an inexpensive medium by a *Bacillus megaterium* strain isolated from marine sediments. *Journal of Environmental Chemical Engineering*, 5(1), pp. 1-9, 2017. DOI: 10.1016/j.jece.2016.11.012
- [20] Bora, L., Das, R. and Gohain, D.A., novel melt stable and high tensile strength biopolymer (polyhydroxyalkanoates) from *Bacillus megaterium* (MTCC10086) and its characterization. *Journal of Basic Microbiology*, 54(9), pp. 1012-1016, 2014. DOI: 10.1002/jobm.201300277
- [21] Balakrishna Pillai, A., Jaya Kumar, A., Thulasi, K. and Kumarapillai, H., Evaluation of short-chain-length polyhydroxyalkanoate accumulation in *Bacillus aryabhatai*. *Brazilian Journal of Microbiology*, 48(3), pp. 451-460, 2017. DOI: 10.1016/j.bjm.2017.01.005
- [22] Shrivastav, A., Mishra, S.K., Shethia, B., Pancha, I., Jain, D. and Mishra, S., Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing *Jatropha* biodiesel byproduct. *International Journal of Biological Macromolecules*, 47(2), pp. 283-287, 2010. DOI: 10.1016/j.ijbiomac.2010.04.007
- [23] Faccin, D.J.L., Martins, I., Cardozo, N.S.M., Rech, R., Ayub, M.A.Z., Alves, T.L.M., Gambetta, R. and Secchi, A.R., Optimization of C:N ratio and minimal initial carbon source for poly(3-hydroxybutyrate) production by *Bacillus megaterium*. *Journal of Chemical Technology and Biotechnology*, 84(12), pp. 1756-1761, 2009. DOI: 10.1002/jctb.2240
- [24] Valappil, S., Misra, S., Boccaccini, A., Keshavarz, T., Bucke, C. and Roy, I., Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised *Bacillus cereus* SPV. *Journal of Biotechnology*, 132(3), pp. 251-258, 2007. DOI: 10.1016/j.jbiotec.2007.03.013
- [25] Sathiyarayanan, G., Saibaba, G., Seghal Kiran, G. and Selvin, J., A statistical approach for optimization of polyhydroxybutyrate production by marine *Bacillus subtilis* MSBN17. *International Journal of Biological Macromolecules*, 59, pp. 170-177, 2013. DOI: 10.1016/j.ijbiomac.2013.04.040
- [26] Baikar, V., Rane, A. and Deopurkar, R., Characterization of Polyhydroxyalkanoate Produced by *Bacillus megaterium* VB89

- Isolated from Nisargruna Biogas Plant. Applied Biochemistry and Biotechnology, 183(1), pp. 241-253, 2017. DOI: 10.1007/s12010-017-2441-4
- [27] Pradhan, S., Dikshit, P.K. and Moholkar, V.S., Production, ultrasonic extraction, and characterization of poly (3-hydroxybutyrate) (PHB) using *Bacillus megaterium* and *Cupriavidus necator*. Polymers for Advanced Technologies, 29(8), pp. 2392-2400, 2018. DOI: 10.1002/pat.4351
- [28] Barham, P.J., Keller, A., Otun, E.L. and Holmes, P.A., Crystallization and morphology of a bacterial thermoplastic: poly-3-hydroxybutyrate. Journal of Materials Science, 19(9), 2781-2794, 1984. DOI: 10.1007/BF01026954
- [29] Rodríguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell-Monfort, M., Braunegg, G. and Marqués-Calvo, M.S., High production of poly(3-hydroxybutyrate) from a wild *Bacillus megaterium* Bolivian strain. Journal of Applied Microbiology, 114(5), pp. 1378-1387, 2013. DOI: 10.1111/jam.12151
- [30] Chaijamrus, S. and Udupay, N., Production and characterization of polyhydroxybutyrate from molasses and corn steep liquor produced by *Bacillus megaterium* ATCC 6748. Agricultural Engineering, [online]. 10, pp. 1-12, 2008. [date of reference April 12th of 2019]. Available at: <https://cigrjournal.org/index.php/Ejournal/article/view/1216/1074>
- [31] Pradhan, S., Borah, A.J., Poddar, M.K., Dikshit, P.K., Rohidas, L. and Moholkar, V.S., Microbial production, ultrasound-assisted extraction and characterization of biopolymer polyhydroxybutyrate (PHB) from terrestrial (*P. hysterothorus*) and aquatic (*E. crassipes*) invasive weeds. Bioresource Technology, 242, pp. 304-310, 2017. DOI: 10.1016/j.biortech.2017.03.117

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Supplemental material

Table S1.
Waste glycerol composition reported by the company *Bio D S.A.*

Property	Unit	Requirement	Method	Result
Appearance	Visual	Report	Visual	Viscous amber liquid
Glycerol content	%	80% min.	USP 35	83.10
Karl Fischer humidity	%	15% max	ASTM E-203	11.50
pH	Units	Report	Internal method Bio D S.A.	6.32
Organic Material no	%	Report	ISO 2464	0.95
Glycerol Ashes	%	10% max.	USP 35	4.44
Salts content as NaCl	%	Report	Internal method Bio D S.A.	4.23
NaOH content	mg/Kg	Report	Internal method Bio D S.A.	Not quantifiable
Methanol content	%	0.1% max	ASTM-D7716-11a	<0.02

Source: *Bio D S.A.* Company

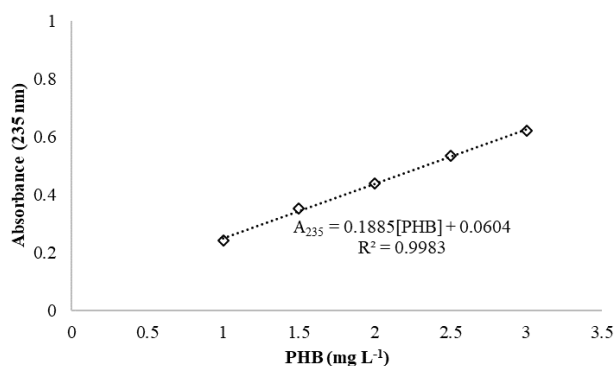


Figure S1. Calibration curve for PHB quantification.

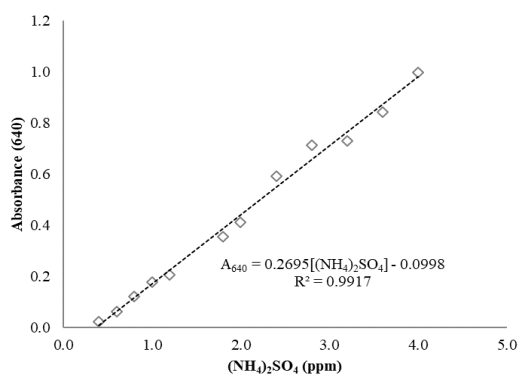


Figure S2. Calibration curve for ammonium quantification.
Source: The Authors.

Source: The Authors.

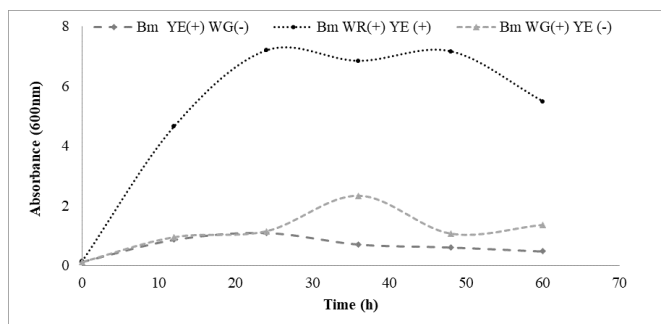


Figure S3. Growth curves of *B. megaterium* LVN01 strains in waste glycerol with and without yeast extract. Bm YE (+) WG (-): *B. megaterium* LVN01 culture in yeast extract without waste glycerol. Bm EL (+) WG (+): *B. megaterium* LVN01 culture in yeast extract with waste glycerol. Bm WG (+) YE (-): *B. megaterium* LVN01 culture in waste glycerol without yeast extract.
Source: The Authors.

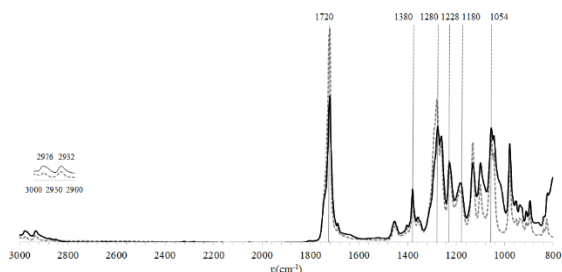


Figure S4. FTIR spectra for PHB commercial (.....) and PHB produced by *B. megaterium* LVN01 from waste glycerol (—).
Source: The Authors.

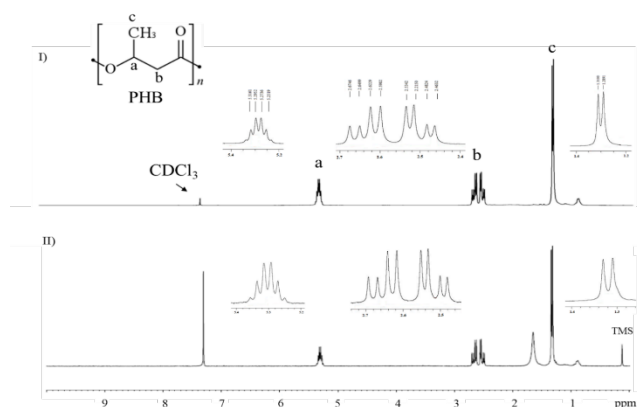


Figure S5. Spectrum 300 MHz ¹H-NMR of (I) PHB commercial compared to (II) spectrum of PHB obtained from *B. megaterium* LVN01.
Source: The Authors.

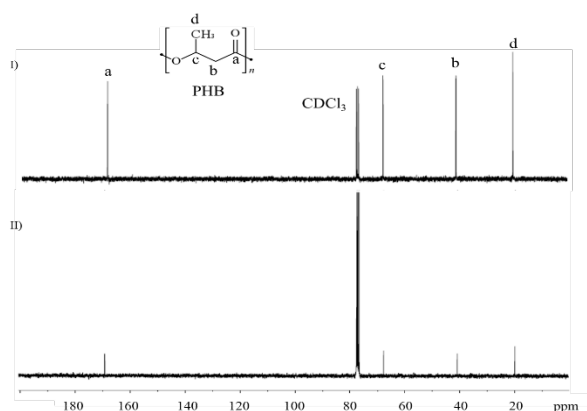


Figure S6. Spectrum 300 MHz ¹³C-NMR of (I) PHB commercial compared to (II) spectrum of PHB obtained from *B. megaterium* LVN01.
Source: The Authors.

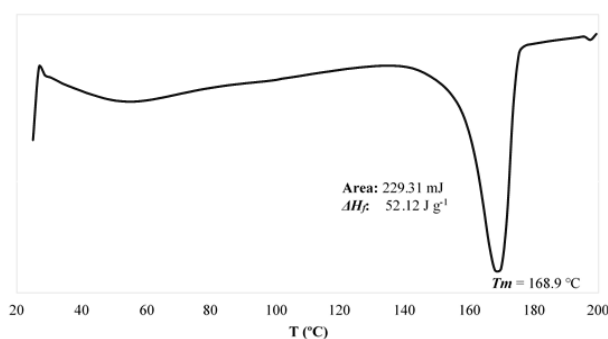


Figure S7. DSC for PHB synthesized by *B. megaterium* LVN01 from waste glycerol.
Source: The Authors.

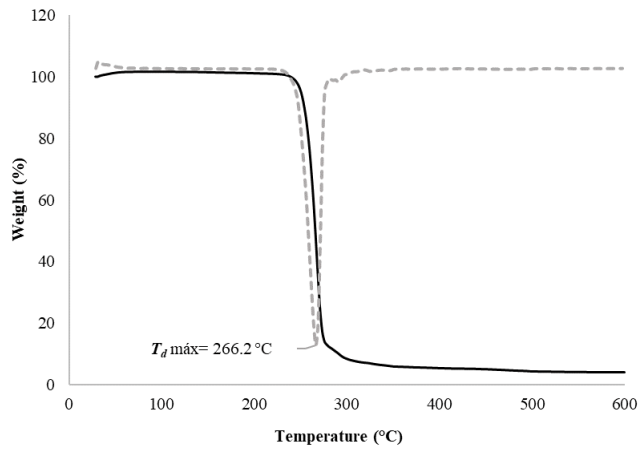


Figure S8. TGA (—) and DTG (-----) for PHB synthesized by *B. megaterium* LVN01 from waste glycerol.
Source: The Author