Production of Polyhydroxybutyrate (PHB) by *Bacillus megaterium* DSM 32 from Residual Glycerol of the Bioenergy Industry

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ABSTRACT: Biodegradable polymers from renewable resources are generating growing interest in the plastic industry because they have properties similar to synthetic polymers. Polyhydroxyalkanoates, mainly polyhydroxybutyrate (PHB), have mechanical and physicochemical properties very similar to their synthetic counterparts. This work explores the use of residual glycerol from the bioenergy industry for the production of PHB by *Bacillus megaterium* DSM 32. The glycerol works as a source of carbon and energy. Raw glycerol was purified with sulfuric acid in order to neutralize saponified fatty acids. The purification process generated three different phases. One of the phases was the glycerol-rich layer; this layer was filtered and concentrated by vacuum distillation process. The purity of the glycerol was determined by thermogravimetric analysis (TGA). Additionally, the physicochemical properties, like viscosity, pH, ash content and density, were measured. The experiments were conducted in shake flasks at 30 °C and 120 rpm. Different glycerol concentrations (20, 30, 40 g/L) were used to evaluate the influence of the initial concentration of glycerol on the biomass accumulation and biopolymer production. The purified glycerol obtained had a high purity (~ 89.5–92.13%); this material does not contain fatty acids, although it contains ~3.7% salts. The final PHB concentration obtained was 0.054 mg/mL.

KEYWORDS: Polyhydroxybutyrate, glycerol, biopolymer, *Bacillus megaterium*, biomass

1 INTRODUCTION

Due to the increase in oil and natural gas prices, and the high environmental impact generated by synthetic plastics, bioplastics are becoming increasingly more competitive with petroleum-based resins [1]. One alternative to bioplastics is the use of biopolymers such as polyhydroxyalkanoates (PHAs). They have the advantage of being biodegradable and can be produced from renewable resources [2].

The PHAs are a family of biopolyesters of 3, 4, 5, 6 hydroxyacids. These compounds are accumulated as intracellular granules. The PHAs can be synthesized by many Gram-positive and Gram-negative bacteria [3]. These compounds represent an alternative source of energy under limited conditions of some essential nutrients like nitrogen, sulfur and phosphorus and under an excess of available carbon [4]. The use of this polymer by the bacteria is considered a survival strategy in changing environments [1].

Poly-3-hydroxybutyrate (PHB) is the most commonly produced PHA [5, 6]. This polymer is of great commercial and environmental interest as a biodegradable plastic material [7] because of its physical properties, which are very similar to those of the petroleum-based plastics. PHB is a thermoplastic that is free from trace catalysts and other chemical compounds, which makes it a nontoxic material, in addition to being biocompatible [8]. Also, PHB is insoluble in water and is resistant to hydrolytic degradation, which differentiates it from other polymers of biological origin. Its permeability to oxygen is very low, which makes it a suitable material for use in the packaging of products sensitive to oxygen [9].

One strategy for improving the production of PHB is the use of strains capable of synthesizing biopolymer

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and reducing the cost by replacement of the carbon source with cheaper ones, like agro-industrial residues or other waste materials [10–12]. Among these carbon sources, some which have been used are raw starch [10], cane molasses [8], soy molasses [13], among others. Glycerol is a co-product of biodiesel production [14, 3, 15–17]. An increase in biodiesel production will significantly raise the amount of crude glycerol in the environment. In contrast to crude glycerol, pure glycerol is a very important raw material within industries such as food, cosmetics and pharmaceuticals, and is also used by microorganisms as a source of energy [18, 19].

Different microorganisms have used glycerol as a source of carbon for the production of PHAs. Among them are *Paracoccus denitrificans* [20–22], *Cupriavidus necator* [23, 24], *Chelatococcus daeguensis* [25], *Novosphingobium* sp. [26] and *Bacillus megaterium* [14, 27]. *Bacillus megaterium* is a Gram-positive bacterium capable of producing PHB under less expensive fermentation conditions using different renewable carbon sources and producing high yields of PHB [8, 27].

Taking this into account, the main objectives of this work were to study the use of purified glycerol, from the production of Costa Rican biodiesel, as a carbon source for the production of PHB by *Bacillus megaterium*, which could substitute polymers derived from petroleum that present environmental problems and low biodegradability.

2 MATERIALS AND METHODS

2.1 Purification of Glycerol

The samples of glycerol were provided by Energías Biodegradables de Costa Rica located in Ochomogo, Cartago. This company produces biodiesel from agricultural raw materials such as vegetable oils (soybean, sunflower, palm, fig, etc.) or used animal fats. In the process of glycerol purification, 200 g of raw glycerol was first conditioned by diluting with 200 ml of distilled water. This step was used to remove the sodium hydroxide residues in the sample. The solution was kept under constant agitation and at a temperature of 30 °C. Once the sample was diluted, the initial pH was between 9 and 10. Subsequently, the dissolution was neutralized with H_2SO_4 at room temperature (98%, Merck, Darmstadt, Germany). Once it reached pH 6.0 the dissolution was transferred to a separation funnel where three distinct phases were observed: a top phase consisting of residual fatty acids from the production of biodiesel, an intermediate glycerol-rich phase and a bottom phase composed of precipitated salts. These phases were carefully separated and the glycerol-rich phase was vacuum filtered using Whatman No. 1 filters (pore size of 11 µm). Then the glycerol was subjected to a vacuum distillation process using a Buchi R-215 rotavapor (Thermo Fisher Scientific, Mississauga, ON, Canada) coupled with a Buchi V-700 vacuum pump (Thermo Fisher Scientific, Mississauga). The operating conditions were: bath temperature 80 °C, vacuum pressure 120 mmbar, cooling water temperature 10 °C, stirring speed 80 rpm, operating time of 2–3 hours. With this process it is possible to concentrate the glycerin and precipitate the rest of the salts that could not be eliminated in the filtration process.

2.2 Characterization of Purified Glycerol

2.2.1 FTIR Spectroscopy Analysis

In order to determine the main functional groups in purified glycerol, Fourier transform infrared (FTIR) analysis was carried out. The FTIR spectra were collected at resolution of 4 cm^{-1} in the transmission mode (4000–400 cm^{-1}) using a FTIR spectrophotometer (Nicolet 6700, Thermo Scientific).

2.2.2 Determination of Purity

The determination of the purity of the purified glycerol was carried out by thermogravimetric analysis (TGA) technique. Approximately 50 mg of sample was used for the TGA analyses and was performed using a TGA Q500 (TA Instruments, USA) with a temperature ramp of 10 °C/min from 0 to 600 °C and subsequently ramped up to 20 °C/min min from 600 °C to 1000 °C under nitrogen (flow rate 90 mL/min). All samples of purified glycerol, crude glycerol and anhydrous glycerol (J.T. Baker, USA) were analyzed. The analyses were performed in triplicate.

2.2.3 Physico-Chemical Analysis

The pH value of the purified glycerol was determined with a Sartorius PB-11 pH meter (Göttingen, Germany). The density of the glycerol was determined using a 25 mL pycnometer and a water bath with a constant temperature of 25 °C.

The viscosity of the glycerol was determined using a Brookfield LVDV-II+P model viscometer (Middleborough, MA, USA) and using a LV 2 spindle. A 25 ml sample was taken and the viscosity was determined under conditions of 25 °C and 100 rpm. The obtained viscosity was reported in centipoise units.

For the ash content, 5 g of purified glycerol sample was then weighed and the crucible-glycerol assembly was heated at 120 °C for 2 hours to boil off the water present in the sample. The remainder was calcined to constant weight at 750 °C for about 25 minutes, after

which the samples were again dried, weighed and the ash mass percentage was calculated.

2.3 Production of PHB

2.3.1 Microorganism

It was used *Bacillus megaterium* DSM 32, which was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

2.3.2 Strain Storage and Adaptation to Glycerol

The *B. megatherium* cells were stored at −70 °C in 1.5 mL cryovials containing 150 µL glycerol and 850 µL of a previously prepared nutrient liquid culture. These cells were used to inoculate nutrient agar plates supplemented with 20 g/L glucose. The plates were incubated at 30 °C for 2 days. The *B. megaterium* cells grown in nutrient broth at 30 °C were used to inoculate nutrient agar plates supplemented with 5 g/L of glycerol and 15 g/L of glucose. Single colonies were used to inoculate nutrient agar plates supplemented with 10 g/L of glycerol and 10 g/L of glucose; another culture was made in a nutrient agar plate containing 15 g/L of glycerol and 5 g/L of glucose; and finally a single colony of this culture was transferred into a medium with 20 g/L of glycerol. In each culture the cells were left 3 days.

2.3.3 Culture Media

The seeding medium was prepared according to Kim *et al.* [28]. It contained 1 g/L $(NH_4)_2S0_4$; 1.5 g/L KH_2PO_4; 3.5 g/L Na_2HP0_4; and 0.2 g/L $MgSO_4$· Different concentrations of purified glycerol were used as carbon sources (20, 30 and 40 g/L). The pH was adjusted to 6.8 using NaOH. The carbon source and the $MgSO_4.7H_2O$ were autoclaved separately and added aseptically to the medium.

2.3.4 Preculture and Inoculum

Precultures (100 mL shake flasks containing 20 mL of the nutrient broth, pH 7.0) were inoculated with a single *B. megaterium* colony from nutrient agar plates and incubated at 30 °C for 24 h on a Heratherm IMH 180 incubator (Thermo Scientific, Langenselbold, Germany) with a rotary shaker at 120 rpm (Daigger Scientific, IL, USA).

2.3.5 Shake Flask Culture

Shake flask experiments were performed in a 125 mL Erlenmeyer flask containing 25 mL of the medium described previously, using different concentrations of glycerol and glucose. The flasks were incubated at 30 °C in an orbital shaker for 36 h. The experiments were carried out in triplicate. Fed-batch fermentation in shake flask with the higher concentration of glycerol was performed, under the same conditions described above, to study the production of PHB. After the cell density reached an absorbance of 3.0, a glycerol solution was introduced manually to keep its concentration constant and thus stimulate the production of PHB. Growth was monitored up to 78 hours; all samples were then collected for analysis, as described in Section 2.4.

2.4 Analytical Methods

The biomass of the culture was determined using optical density measurements at 600 nm in a UV-1800 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). The PHB content of the biomass from the *B. megaterium* cultivation was dissolved in 10 ml chloroform and 1 ml of this solution was transferred to a fresh sample glass tube. Once the chloroform in the sample tube was completely vaporized, 10 ml of concentrated H_2SO_4 was added and the tube sealed with a glass stopper. This tube was heated in a boiling water bath for 20 minutes to complete the conversion of PHB into crotonic acid. The sample was cooled, mixed vigorously with a vortex mixer and transferred to a quartz cuvette for measurement of UV absorbance in a spectrophotometer. Commercial PHB (Sigma-Aldrich, St. Louis, MO, USA) was converted into crotonic acid by the method previously described. The presence of PHB was confirmed by the presence of a peak obtained at 235 nm. The UV absorbance of the commercial PHB was used to generate a standard curve and determine the concentration of PHB from *B. megaterium* cultures.

2.5 PHB Extraction

The purification method used was proposed by Hahn *et al.* [29]. The PHB containing bacteria was collected by centrifugation at 5000 rpm for 15 min. The biomass was dried in a drying oven at 50 °C for 24 h. The dry biomass was treated with dispersions of 2.5 mL chloroform and 2.5 mL of sodium hypochlorite solution 20% (v/v). The mixture was incubated at 35 °C for 1 h with constant agitation, and then the mixture was centrifuged at 4000 rpm for 10 min. Were obtained three separated phases. The upper phase was hypochlorite solution, the middle phase contained cell debris and undisrupted cells, and the bottom phase was chloroform containing PHB. The hypochlorite solution phase was removed with a pipette, and then the chloroform

phase was obtained by filtration with a 0.22 µm pore size Ministart® membrane filter. Then, the chloroform phase was evaporated completely to obtain the purified PHB.

2.6 Data Analysis

The statistical program Minitab 17 (version 2.9; Minitab Inc., State College, PA, USA) was used to perform the analysis of the results. Data were analyzed by using one-way analysis (ANOVA). P values of < 0.05 were considered significant.

3 RESULTS AND DISCUSSION

3.1 Purification of Crude Glycerol

The production of PHB from crude glycerol starts with the glycerol purification process before the fermentation step by chemical methods. Glycerol is a carbon source that has been used for the production of PHB in bacteria such as *Ralstonia eutropha* [17], *Novosphingobium capsulatum* [26], *Chelatococcus daeguensis* [25], different *Bacillus* species [14, 27], and mixed microbial community [30]. However, it has been reported that PHB production increases with the use of purified glycerol [31].

The content of purified glycerol varied between 88.8% and 91.1% (Table 1). The process of purification involved the acidification of the crude glycerol, which resulted in the formation of three phases: a top phase of fatty acid, a middle glycerol-rich phase and a bottom phase of the remaining inorganic salts of the biodiesel production process. The use of pH adjustment to remove saponified fatty acids from a crude glycerol solution has been reported previously and has a very important effect on the final composition of the purified glycerol [32, 33]. The percentages of purified glycerol obtained in this work are higher than those reported by Ooi *et al.* [34], where the percentage of glycerol was 51.4%, and Simpson *et al.* [35], where the percentage was 82.5%; and is similar to that of the bipolar electrodialysis method used by Schaffner *et al.* [36], in which the percentage obtained was 95%. However, the disadvantages of bipolar electrodialysis are that the yield depends mainly on the concentration of impurities in the crude glycerol and it is also a more expensive technique. Impurities such as fatty acids and Na^+ ions can be deposited on the membrane and could decrease the efficiency of purification [37].

Table 1 shows the physicochemical properties that were obtained from each of the monthly glycerol samples.

In this work, a pH value of 6 for the acidification step resulted in total ash content between 3 and 4% (Table 1), similar to that reported by Manosak *et al.* [38]. This ash content may be due to the content of SO_4^{-2} ions, resulting from the ionization of the H_2SO_4 added during the acidification step, which is complexed with the sodium ions of the salts present in the crude glycerol, thus forming Na_2SO_4 that is relatively insoluble in aqueous solution, especially at a low pH; consequently, these salts precipitate during the phase separation process and distillation process [38]. Regarding the physicochemical properties of the purified glycerol, we only found significant differences in pH and viscosity. This may be due to the fact that both the pH value and the viscosity are properties that can be affected by the water content in the sample.

3.2 Thermogravimetric Analysis of the Glycerol

Thermogravimetric analysis (TGA) of the glycerol was used to determine the purity of purified glycerol. The TGA analysis showed that the decomposition of the crude glycerol presented four phases. In Figure 1 the weight loss curve of the crude glycerol with 10 °C/min of temperature flow can be observed. The weight loss values during phase 1 were 14.2%, which may be due to substances such as methanol and water present within the production of biodiesel. Within phase 2 there is a 56.2% weight loss at a maximum temperature of 198.0 °C, which indicates the volatilization of the glycerol present in the sample [39]. In phase 3 a 22.5% mass loss was obtained, which is represented by impurities of fatty acids that did not react in the transesterification [39]. And finally, there was a 5.9%

Table 1 Physicochemical properties of the purified glycerol.

Parameter					
Time (month)	**Density (g/mL)**	**pH**	**Viscosity (cP)**	**Ashes (% p/p)**	**Glycerol content (% p/p)**
1	1,18[a]	6,61[a]	470[a]	3,03[a]	88,80[b]
2	1,19[a]	6,7[b]	465,5[b]	3,24[a]	91,53[a]
3	1,19[a]	6,48[c]	530[c]	3,89[a]	92,13[a]

*Values with the same letter do not show significant statistical differences.

Table 2 Analysis of the TGA curve; results obtained from each phase on the TGA curve of crude and purified glycerol.

	Crude glycerol				Purified glycerol
Phases	**1**	**2**	**3**	**4**	**1**
Mass lost (%)	14.2	56.2	22.5	5.9	89.5
Initial mass loss temperature (°C)	30	87.5	324.1	397.1	116
Maximum mass loss temperature (°C)	65	198	355.4	481.5	231.1
Final mass loss temperature (°C)	86.2	300.8	396.2	532.7	303.9

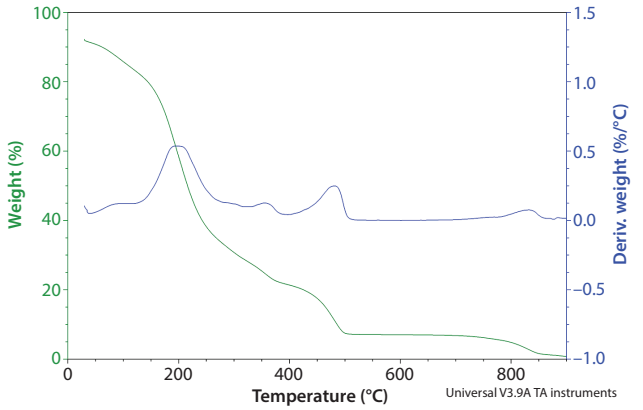

Figure 1 TGA mass loss curve of crude glycerol with 10 K/min.

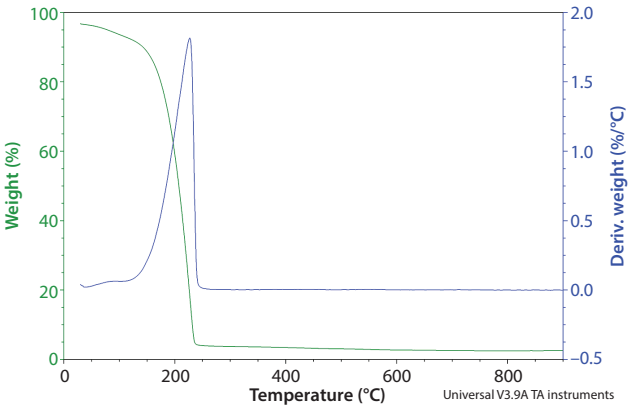

Figure 2 TGA mass loss curve of purified glycerol with 10 K/min.

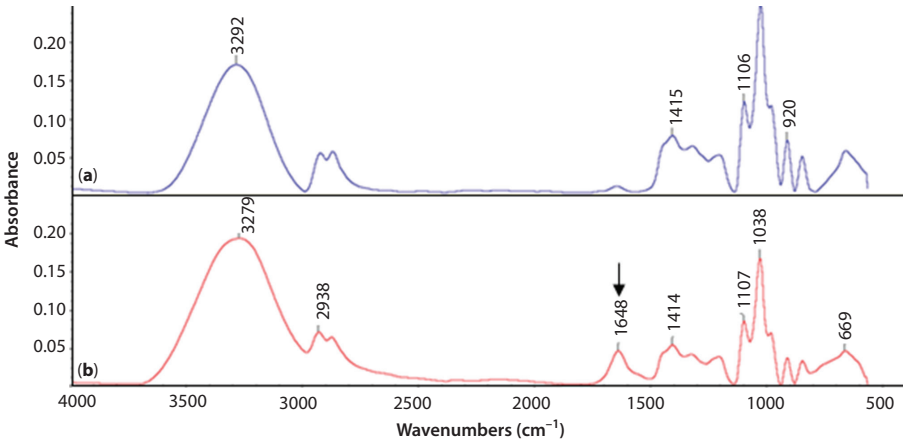

Figure 3 Commercial glycerol representative FTIR spectrum (**a**), and the purified glycerol (**b**) with the acidification step performed at pH 6.0.

loss in phase 4, which could be the salts derived from the catalyst used in transesterification [39].

Table 2 shows the mass loss, the initial mass loss temperature, the maximum mass loss temperature and the final mass loss temperature for each phase of the crude and purified glycerol obtained from the TGA curve (Figures 1 and 2).

The TGA curve of the purified glycerol obtained in August, presented in Figure 2, only presents one phase, which represents an 89.1% weight loss beginning at 115.7 °C and ending in 258.5 °C. The residual mass obtained was 3.7%, indicating salt impurities, which is a very similar value to that obtained for the ash content showed in Table 1.

3.3 FTIR Analysis

The comparative FTIR between the purified glycerol and the glycerol J.T.Baker® used as a reference is seen in Figure 3. Characteristic peaks can be observed

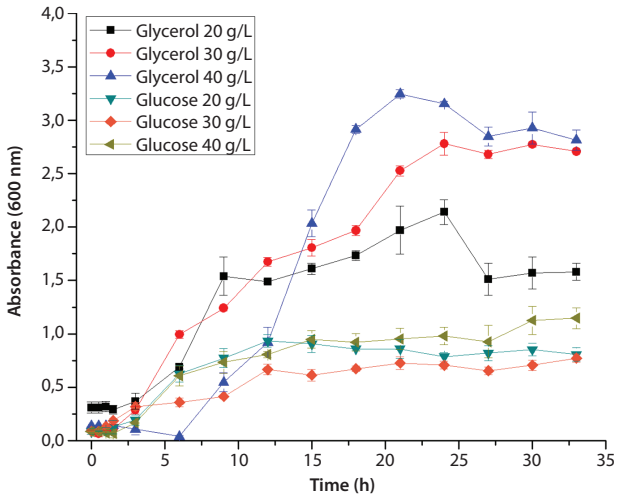

Figure 4 Growth curve of *B. megaterium* under different concentrations of purified glycerol and glucose.

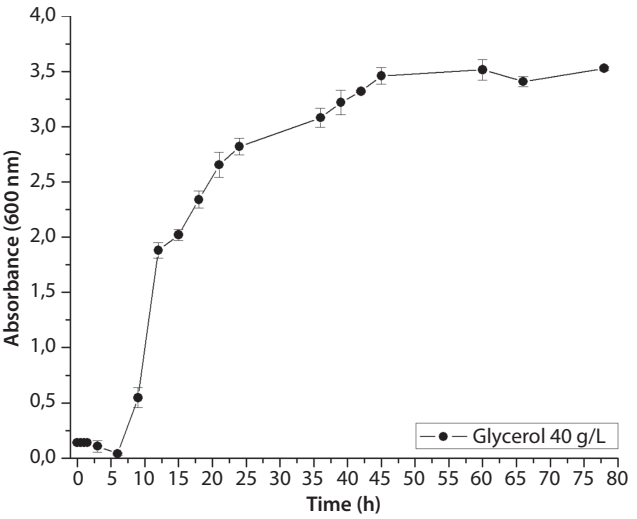

Figure 5 Growth curve of *B. megaterium* under a fed batch in shake flask culture using 40 g/L of purified glycerol.

such as stretching of the O-H bond at 3279 cm^{-1}, C-H stretching at 2938 cm^{-1}, C-O-H bond bending at 1414 cm^{-1}, O-H bending at 920 cm^{-1} and also O-H bending at 1648 cm^{-1} of free water [16]. It can be seen that the signals obtained in the FTIR of the purified glycerol coincide with those obtained in pure glycerol; however, the marked signal located at 1648 cm^{-1} is not found in the pure glycerol spectrum (a), indicating that the water content within the sample of purified glycerol is very high compared to commercial glycerol. There was no presence of fatty acids in the purified glycerol, since these compounds present characteristic bands at 1580 and 1740 cm^{-1}, whereas the band at 1580 cm^{-1} is related to the carboxylate (COO^-) and at 1740 cm^{-1} is due to the presence of the carbonyl group (C=O) characteristic of esters or carboxylic acids; this could also be confirmed in the TGA analysis that was performed on purified glycerol (Figure 2) [40]. This phenomenon is due to the fact that in the presence of acidic conditions, the soap residues present in the crude glycerol were hydrolyzed to insoluble free fatty acids according to the following reaction [38, 41]:

$$RCOONa + H_2SO_4 \rightarrow RCOOH + Na^+ + SO_4^{-2} \quad (1)$$

We can conclude that the purification process used in this work was very effective because of the higher purification percentages obtained, and could be used not only for the purification of glycerol generated from transesterification of waste oils but also for crude glycerol from biodiesel production using other oils such as palm, castor and *Jatropha curcas*.

3.4 Fermentation Procedure

3.4.1 *Batch Culture in Shake Flasks*

Batch cultures in shake flasks were conducted under nutrient-rich conditions to determine the bacterial growth rate. A growth curve was determined for glucose and compared with that of glycerol derived from the biodiesel production (Figure 5). These results indicate that compared to glucose, the purified glycerol is a good carbon source for the growth of *B. megaterium* DSM 32, which could result in higher PHB productivity. This might be due to the fact that glycerol is metabolized by the β-oxidation pathway generating more ATP molecules compared to the glycolysis pathway, a process by which glucose is metabolized, which could stimulate more growth of the bacteria [42–44]. In Figure 4 it can be observed that with the concentration of 40 g/L glycerol we obtained the highest absorbance, reached at 21 hours.

3.4.2 *Fed-Batch Culture in Shake Flask*

A fed-batch culture in shake flasks with an initial concentration of 40 g/L of glycerol was carried out to determine the cell growth rate and the production of PHB. It consisted of an initial cell growth phase under nutrient-rich conditions, followed by PHB formation under nutrient limitation and an excess of carbon source [45]. The concentration of 40 g/L has also been reported by other authors for the growth and accumulation of the polymer [25]. Figure 5 shows the growth curve obtained after addition of glycerol at 21 hours. It was determined that the fed-batch fermentation in shake flask improves the growth, which could increase

the PHB accumulation. This strategy has been used to enhance the polymer accumulation compared with the batch fermentation [2, 22, 25]. Cavalheiro *et al.* [15] determined that before nitrogen depletion, glycerol is mainly directed towards cell production and maintenance and after nitrogen depletion the glycerol is mainly used for PHB production in the stationary phase, with no further increase in the residual biomass. This is in line with our results, where in the stationary phase we found the maximum production of PHB without cell growth. Similar results have been reported by Naranjo *et al.* [27], who observed that the highest accumulation of PHB was at the beginning of the stationary phase at around 40 hours of fermentation, which is very similar to what was obtained in this study.

The study focused on the use of an economic culture medium, using purified glycerol from the biodiesel industry in Costa Rica as a source of carbon, and without the addition of microelements. We obtained a PHB concentration of 0.054 mg/mL in the stationary phase (Figure 6). The genus *Bacillus* has also been used by other authors for the production of PHB. Full *et al.* [13] obtained a PHA concentration of 1.41 mg/mL using glycerol as a carbon source in *Bacillus* sp. strain CL1. Kulpreecha *et al.* [8] found, in *Bacillus megaterium* strain BA-019, a concentration between 0.55–3.90 mg/mL using sucrose and molasses as carbon sources; and Rodríguez-Contreras *et al.* [14] reported a concentration of 2.35 g/L of PHB using *Bacillus megaterium* uyuni S29 and commercial glucose. Other strains have been reported as producers of PHB using glycerol as a carbon source, including *Ralstonia eutropha* H16, *Paracoccus denitrificans* and *Cupriavidus necator,* achieving concentrations of 1.08, 0.33 and between 0.02–1.10 g/L respectively. Therefore, further research is required to enhance the production of PHB, one strategy could be adding microelements in the culture media to increase the biomass of the *Bacillus megaterium* DSM 32.

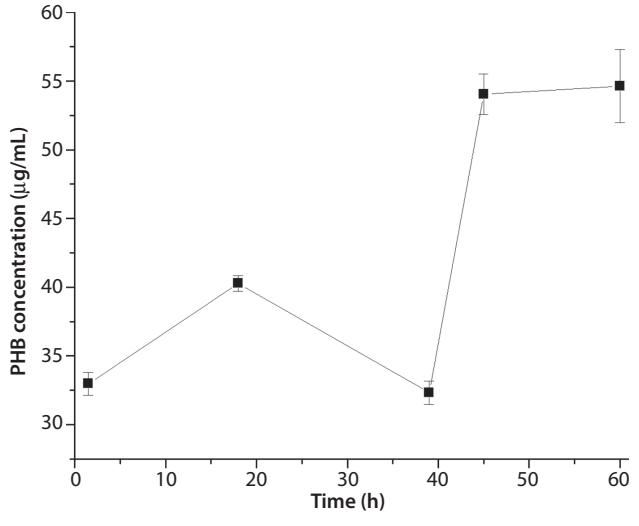

Figure 6 Growth curve and PHB accumulation by *B. megaterium* under a fed-batch fermentation in shake flask.

The PHB extraction and purification method using sodium hypochlorite and chloroform has the advantage that it is very simple and efficient. In previous studies, Hahn *et al.* showed that this purification technique reduces the degradation of PHB by hypochlorite and reduces many problems of other extraction methods [29]. This is due to the fact that once the hypochlorite lyses the cell wall, the polymer is released into the medium and dissolved in chloroform immediately, which provides a protective function to the PHB.

The method of quantification used [46] is based on the rupture of PHB polymer in its monomer units, which reacts with sulfuric acid. A dehydration reaction converts the monomeric units of hydroxybutyric acid to crotonic acid, which is capable of absorbing UV radiation at a wavelength of 235 nm. The amount of UV radiation absorbed by the crotonic acid is directly proportional to the amount of PHB present in the sample.

4 CONCLUSIONS

Due to its availability and low price, glycerol should be considered as a suitable inexpensive carbon source for PHB production, as well as the specie *B. megaterium*, which is able to use this residue as an energy source for its development and accumulation of the polymer. We determined that the fed-batch fermentation in shake flask improves the growth and the PHB accumulation in the stationary phase. The quantification method used was adequate to determine PHB production at laboratory scale, specifically at flask scale.

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