Research Article

Production and Characterization of Polyhydroxyalkanoates and Native Microorganisms Synthesized from Fatty Waste

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Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible plastics. They are synthesized by a wide variety of microorganisms (i.e., fungi and bacteria) and some organisms such as plants, which share characteristics with petrochemical-based plastics. The most recent studies focus on finding inexpensive substrates and extraction strategies that allow reducing product costs, thus moving into a widespread market, the market for petroleum-based plastics. In this study, the production of polyhydroxybutyrate (PHB) was evaluated using the native strains, *Bacillus megaterium*, *Bacillus* sp., and *Lactococcus lactis*, and glycerol reagent grade (GRG), residual glycerol (RGSB) byproduct of biodiesel from palm oil, Jatropha oil, castor oil, waste frying oils, and whey as substrates. Different bacteria-substrate systems were evaluated thrice on a laboratory scale under different conditions of temperature, pH, and substrate concentration, employing 50 mL of broth in 250 mL. The bacterial growth was tested in all systems; however, the *B. megaterium* GRG system generated the highest accumulation of PHA. The previous approach was allowed to propose a statistical design optimization with RGSB (i.e., RGSB, 15 g/L, pH 7.0, and 25°C). This system reached 2.80 g/L of PHB yield and was the optimal condition tested; however, the optimal biomass 5.42 g/L occurs at pH 9.0 and 25°C, with a substrate concentration of 22 g/L.

1. Introduction

Biopolymers are macromolecules produced by living cells from renewable sources, which give rise to known biodegradable plastics and therefore can be an alternative to solve environmental and social problems generated by the plastics industry from petrochemicals.

The petroleum-based plastics are highly resistant to temperature, pressure, chemical solvents, UV light, among other factors, which are widely employed in all fields of industry. For example, while in 1950 1.5 million ton (mTon) of plastics was produced, in contrast in 2010 250 mTon is reported and is expected to reach 330 mTon 2015 [1]. The plastics production scale (with an annual increase close to 6.5% during the next 5 years) is exponential accumulation of plastic waste in the

environment [2] and pollution of water bodies and soils. This has a negative impact on human health, turning them into a social problem of high interest [3].

Therefore, researchers around the world have focused on the production of biodegradable polymers in the recent decades, using different methods and procedures, mainly in the family of the polyhydroxyalkanoates (PHA) and its derivatives thereof (e.g., polylactic polyesters aliphatic, polysaccharides) [4].

PHAs are biodegradable biopolyesters completely synthesized and catabolized by a wide range of microorganisms (i.e., bacteria and fungi) and some plants [5, 6]. The bacterial PHA is intracellularly synthesized as cytoplasmic bodies hydrophobics, usually in excess of carbon source and deficiency of other essential nutrients, such as O, N, P, S,
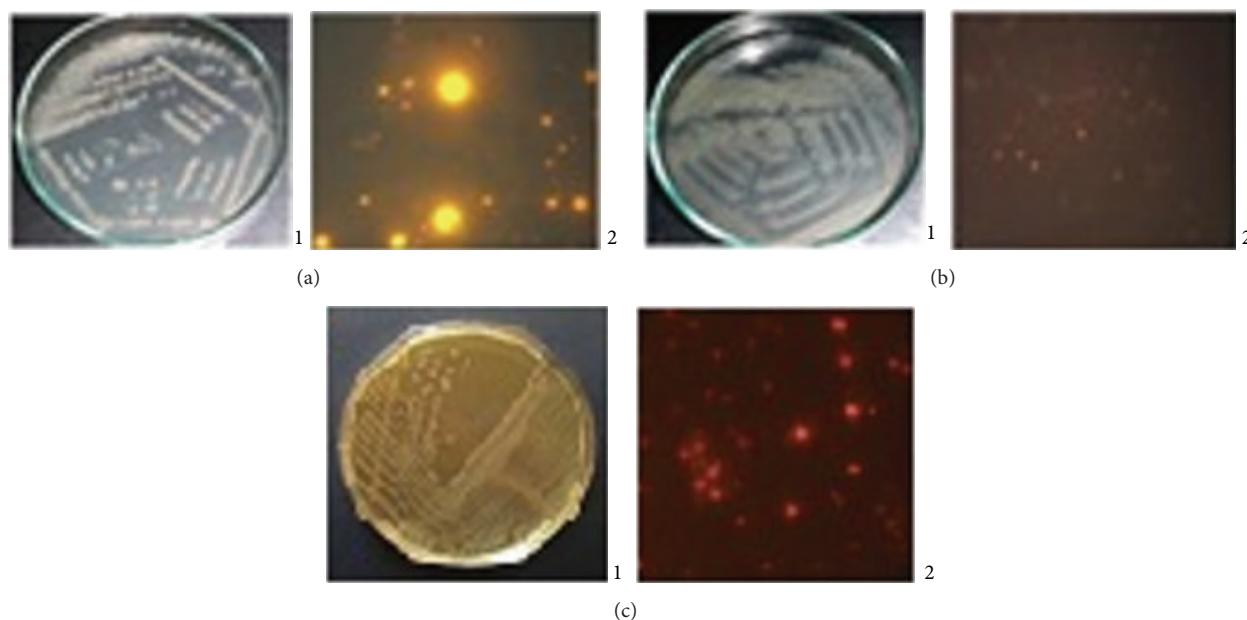
(a)

(b)

(c)

Figure 1: Macro- and microscopic morphology of the colonies of strains: (a) *B. megaterium*, (b) *Bacillus* sp., (c) *L. lactis*. 1, macroscopic characteristics of the colonies, 2, fluorescence microscopy at 450 nm with Blue Nile [23, 24].

or Mg [7]. However, the production cost is relatively higher compared to their petrochemical analogs, which have limited the industrial production of PHA. For example, the cost of production of PHB (one of the most studied type of PHA) was estimated at US 7.8–11.2/kg, which corresponds to 30–40% carbon source; values exceed the prices of similar polymers obtained from oil (i.e., US 1.45 per kilogram in 2014) [2, 8, 9].

It clear that seeking a profitable production on an industrial scale depends on several factors such as the ability of the microorganism to employ a source of inexpensive carbon (recently, attention has focused on farm wastes and industrial byproducts), cost of the means of culture, growth rate, polymer synthesis speed, quality and quantity of PHA, and the cost of subsequent processes for the production of plastic [2, 9]. Furthermore, huge efforts are focused on finding renewable and inexpensive raw materials [10, 11], in the evaluation of genetically modified organisms [12], in the use of microbial consortia [13, 14] to reach improvements in the process of extraction and purification [15–17], in the development of technologies in transgenic plants [18, 19], and in the variation in strategies fermentation process (i.e., batch, fed batch, and continuous) [20–22].

In Colombia several companies (e.g., food and energy) generating products that can be exploited as potential sources of carbon in the production of PHAs exist. Thus, this study was focused on evaluating the potential of native strains for producing PHAs [25–27] from inexpensive substrates, waste, or byproducts from different industries (i.e., Jatropha oil, castor oil, waste frying oil, whey, and residual glycerol).

2. Materials and Methods

2.1. Bacterial Strains. Three native strains were employed in this study (Figure 1) from Collection of PROBIOM

(Production Structure and Application of Biomolecules) research group, isolated from different industrial waste, and selected based on biochemical, molecular, and analytical tests for their ability to produce PHA.

   (i) *Bacillus megaterium* and *Bacillus* sp. were isolated from soil contaminated with sisal juice by Sánchez Moreno et al. [28], employing microbiological (serial dilutions), molecular (PCR amplification and sequencing of a portion of the small subunit 16S rDNA), and chemical techniques (determination of PHA monomers by GC/MS), to characterize and determine potential strains in biopolymer synthesis.

  (ii) *Lactococcus lactis* was isolated from whey by Cardona et al. [29], employing microbiological (serial dilutions), molecular (PCR amplification and sequencing of a portion of the small subunit 16S rDNA), and chemical techniques (determination of PHA monomers by GC/MS), to characterize and determine potential strain in biopolymer synthesis.

The potential of the strains to produce PHA from sugar waste was evaluated by Salazar [23].

2.2. Culture Media. Several culture media supplemented with carbon sources of different nature and origin (i.e., Jatropha oil, castor oil, waste oil frying, whey, glycerol, and residual glycerol) were tested with each microorganism.

2.2.1. Whey Culture Medium as a Carbon Source. Minimal Salts Medium (MSM) was supplemented with KH_2PO_4 1.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 11.0 g/L, NH_4Cl 16 g/L, $MgSO_4 \cdot 7H_2O$ 1.4 g/L, 1 mL of trace element solution, $FeSO_4 \cdot 7H_2O$ 10 g/L, $ZnSO_4 \cdot 7H_2O$ 2.25 g/L, $CuSO_4 \cdot 5H_2O$ 1.0 g/L, $MgSO_4 \cdot 5H_2O$

Table 1: Fatty acid composition (%) of Jatropha and castor oils [30–32].

% Fatty acids	Oil		
	Jatropha		Castor
	[30]	[31]	[32]
Saturated			
Myristic C14:0	<0.1	0.1	—
Palmitic C16:0	13.2–13.54	12.11	1.0–2.0
Margaric C17:0	0.1–0.11	—	
Stearic C18:0	7.23–7.34	6.02	1.0–1.5
Dihydroxystearic C18:0	—	—	0.7–1.0
Arachidic C20:0	0.2–0.22	—	—
Behenic C22:0	<0.1	—	—
Lignoceric C24:0	<0.1	—	—
Unsaturated			
Palmitoleic C16:1	0.76–0.81	0.75	—
Ricinoleic C18:1	—	—	85.0–89.5
Oleic C18:1	47.04–48.17	35.74	3.0–3.5
Eicosenoic 20:1	<0.1	—	0.3–0.5
Linoleic C18:2	29.17–30.41	44.93	3.0–4.2
Linolenic C18:3	0.2–0.22	0.34	0.2–0.8

0.5 g/L, $CaCl_2 \cdot 2H_2O$ 2.0 g/L, $Na_2B_4O_7 \cdot 10H_2O$ 0.23 g/L, $(NH_4)_6Mo_7O_{24}$ 0.1 g/L, 10 mL of HCl 35%, and whey 20 g/L. The medium was supplemented with yeast extract (1 g/L) and adjusted to pH 7.0.

To avoid the eventual presence of proteins, the broth was acidified with HCl till pH values were lower than 4.0, prior to the addition of the whey, and thereafter it was sterilized. When the whey reached room temperature, it was centrifuged at 10,000 rpm for 15 min. The supernatant was filtered to remove floating particles, by fast filter paper Whatman (Red Band, Grade 589/3, diameter 125 mm). The volume concentration was adjusted at 20 g/L in the culture medium [24].

2.2.2. Culture Media Oils (Castor, Jatropha, and Waste Frying) as Carbon Sources. For growth of bacterial strains MSM was employed containing KH_2PO_4 1.2 g/L, $Na_2HPO_4 \cdot 12H_2O$ 11 g/L, NH_4Cl 16 g/L, $MgSO_4 \cdot 7H_2O$ 1.4 g/L, and 1 mL of trace element solution, prepared as was described in Section 2.2.1. The medium was adjusted at pH 7.0 before the addition of the oil (i.e., castor, Jatropha, or waste frying), at 20 g/L, and was supplemented with yeast extract 1 g/L. Jatropha and castor oils used in this work were from Colombia; the first one has higher proportion of oleic and linoleic acids, followed by palmitic and stearic acids; meanwhile this castor oil has high content of ricinoleic acid (Table 1).

Although the frying oil used in this work was not characterized, it is known that this kind of oil is rich in free fatty acids (mono- and diacylglycerol), total polar material (oxidized monomeric and dimeric and oligomeric triglycerides), and compounds such as aldehydes and ketones and polymerized triglycerides (dimeric and polymeric triglycerides with ring structure) [33].

2.2.3. Culture Media with Commercial Residual Glycerol and Glycerol as Carbon Sources. Two samples of glycerol as carbon sources were employed, glycerol GR (99.5% v/v) and glycerol produced in the biodiesel industry (81.6% v/v). The culture contains glycerol GR (or the residual one (GRSB)) 10 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 15 g/L, $Na_2HPO_4 \cdot 12H_2O$ 9 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, and 1 mL of trace element solution (for further information see Section 2.2.1). Similarly, the medium was supplemented with yeast extract 1 g/L and adjusted at pH 7.0. According to Cavalheiro et al. [3], the carbon source was autoclaved separately before being added to the medium.

2.3. Terms of Microbial Growth. The bacterial growth was evaluated employing different substrates as carbon source, in a shaker Innova™ 4400. In all cases 250 mL Erlenmeyer flasks were employed, with a working volume of 50 mL, which was considered 10% v/v inoculum for.

2.3.1. Fermentations with Whey as a Carbon Source. All the strains tested were incubated at 30°C and 150 rpm [23]. The selection of the "more promising" bacteria-substrate system was according to dry biomass and the presence of PHA, produced under the same operating conditions (30°C, pH 7.0, and 150 rpm). The bacterial growth was monitored at different times (i.e., 0, 24, 36, 48, and 72 h) by triplicate. Sampling points were selected according to previous evaluation of these systems [27, 34–36].

The chemical identification of PHA extraction biomass samples was carried out by GC/MS obtained for each strain.

2.3.2. Fermentations with Oils (Jatropha, Castor, and Frying) and Glycerol (GRG) as Carbon Sources. To choose the best bacteria-substrate system, a 4×3 factorial experimental design was developed; four factors (four carbon sources: castor, Jatropha, waste frying, and GRG) and 3 levels (3 microorganisms: *B. megaterium*, *Bacillus* sp., and *L. lactis*), with series of three replicates, keep the reaction time (48 h), temperature (30°C), pH 7.0, stirring speed (200 rpm), substrate concentration (20 g/L), and volume of culture medium (50 mL) constant. The response variables were the amount of biomass (g/L) and PHA accumulation (g/L).

The chemical identification of the compounds in the samples obtained was carried out by GC/MS for each strain; likewise the biomass produced in fermentation processes was estimated. However, only those extracts displaying greater weights were subjected to analysis by GC/MS.

2.3.3. Fermentations with Residual Glycerol (RGSB) as a Carbon Source. GRSB assays were performed following the protocol described for GRG, but with a fermentation time of 72 h, taking samples (in triplicate) for determination of biomass at 0, 8, 12, 24, 36, and 72 h, respectively.

2.4. Operational Conditions for the Production of PHA in Bacteria-Substrate System. To establish, on a laboratory scale, the best operating conditions for bacteria-substrate system, a factorial experimental design was performed using response surface analysis with series of three repetitions. The system
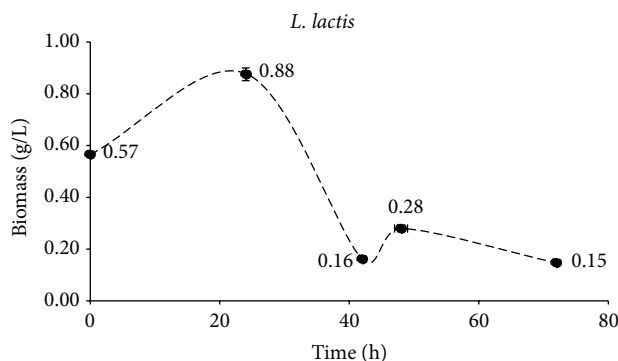
Figure 2: Growth of *L. lactis* employing whey as carbon source.

Table 2: Levels for each factor evaluated in PHA production.

Level	Factors		
	T (°C)	pH	Substrate concentration (g/L)
1	25	5	8
2	30	6	10
3	35	7	15
4	—	8	20
5	—	9	22

response variables were dry biomass production per unit volume, PHA concentration per unit volume, and yield ($Y_{p/s}$). The constants were reaction time 48 h, the agitation 200 rpm, and the volume of the culture medium 50 mL.

Each response variable was studied under the proposed experimental design described in Table 2.

2.5. Analysis Methodologies. The selection of the best bacteria-substrate system was performed by the determination of microbial growth (dry biomass) and chemical parameters (PHA concentration).

2.5.1. Determination of Dry Biomass. The culture media were centrifuged at 8000 rpm and 20°C, 5 min in a JOUAN MR22 Refrigerated centrifuge. The pellet was resuspended in Tris-HCl buffer pH 7.0 and frozen at −70°C for subsequent lyophilization (−50°C and 0.05 mBar). The final powder was weighed with the aim to determine biomass. Media with oil were centrifuged with *n*-hexane (8000 rpm, 20°C, 5 min) to separate the biomass from oleaginous medium.

2.5.2. Extraction and Chemical Characterization by GC/MS of PHA. PHAs were extracted from the bacterial biomass using dispersions of chloroform (99% v/v) and sodium hypochlorite (5% w/v) [37]. 1 mL of each reagent per 20 mg of dry biomass was added. The samples were shaken at 30°C and 200 rpm for 1 h and centrifuged (5 min, 8000 rpm at room temperature) to separate the organic phase, enriched with PHA. Thereafter methanol (95% v/v) was added dropwise to the organic extract until precipitation of PHA. The samples were kept at 4°C, after 12 h, and then the supernatant and the pellet were washed several times with methanol. Finally the polymer was subjected to drying at 60°C and was weighed.

Biopolymer characterization was performed at the Laboratory of Chromatography of the University Industrial of Santander, by gas chromatography with mass selective detector operated in the mode Selective Ion Monitoring (GC-MS/SIM). The quantification was carried out by Standard ISO 5509, 2000, which requires derivatization to the corresponding methyl esters biopolymer. The column employed for analysis was DB-WAX (60 m × 0.25 mm × 0.25 μmi). The injection was performed in splitless mode (Inyection volume = 1 μL). PHB (19.6 g) was employed as reference standard and benzoic acid as internal standard.

3. Results and Discussion

3.1. Selecting the Bacteria-Substrate System. The selection of the best bacteria-substrate system was made based on dry biomass and the amount of PHA produced in each of the fermentations broths.

3.1.1. Evaluation of Bacteria-Whey System. The evaluation of the native strains in media containing whey triggers in the maximum biomass production at 24, 48, and 36 h, for *L. lactis*, *Bacillus* sp., and *B. megaterium*, respectively.

As shown in Figure 2 the major biomass production in the *L. lactis*-whey system occurred at 24 h, with values of 0.88 ± 0.04 g/L.

The microorganism displayed an exponential growth tendency between 0 and 24 h.

In the case of *Bacillus* sp.-whey system, the highest biomass value (0.22 ± 0.02 g/L) occurred at 48 h and represented only a quarter of the biomass obtained with *L. lactis* in just 24 h (Figure 3).

The low amount of biomass generated by this system could be related to low capacity of *Bacillus* sp. to absorb the whey. In this system, the step of adapting the microorganism into the medium was slower than *L. lactis* on the same substrate and under the same conditions, and their growth phase began after 30 h, followed by a decrease between 48 and 72 h.

Finally, the system with *B. megaterium*-whey is illustrated in Figure 4. In this case, the tendency of microbial exponential growth is observed from time zero with a maximum
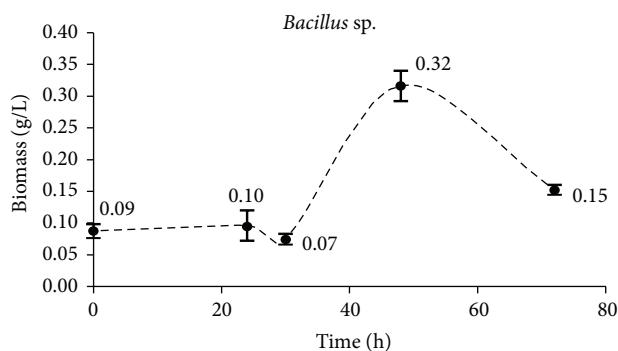
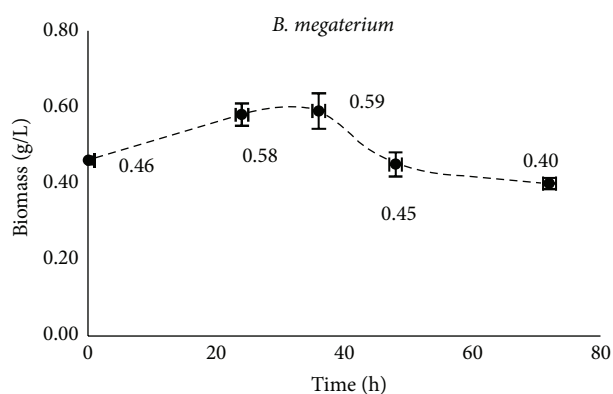FIGURE 3: Growth of *Bacillus* sp. employing whey as carbon source.

FIGURE 4: Growth of *B. megaterium* employing whey as carbon source.

FIGURE 5: Dry biomass obtained in different systems (microorganism substrate).

biomass (0.59 ± 0.06 g/L) at 36 h, whereas between 36 and 72 h a phase decay is seen, reaching biomass values 0.40 ± 0.05 g/L at 72 h.

In contrast, the highest concentration of biopolymer was detected in the *B. megaterium*-whey system. Despite the greater biomass concentration (0.88 ± 0.04 g/L) in the system containing *L. lactis*, the biopolymer production by this microorganism was really low (detected at trace concentrations). This behavior could be related with the growth of *L. lactis* which is based on the metabolic degradation of lactose, substrate barely assimilated by microorganisms which lack lactase enzyme responsible for its degradation.

According to the results, the combination *B. megaterium*-whey was the best system. However, biomass values obtained in the all systems evaluated are far from the values of biomass (3.1 g/L, 36 h) obtained with *B. megaterium* in a broth enriched with glucose and operated under the same conditions (i.e., 30°C, pH 7.0, substrate concentration 20 g/L, and 150 rpm) [23] and obtained by Obruca et al. [34] in *B. megaterium*-cheese whey system (2.82 g/L, 30 h) (with optimized carbon source).

The whey can be reported as carbon source in the production of biopolymers from *B. megaterium* [31, 33], although in this case it did not display good performance, eventually caused by contamination with other byproducts of the processed cheese that develops in the dairy plant which was sampled (the variations in the composition of whey cheese
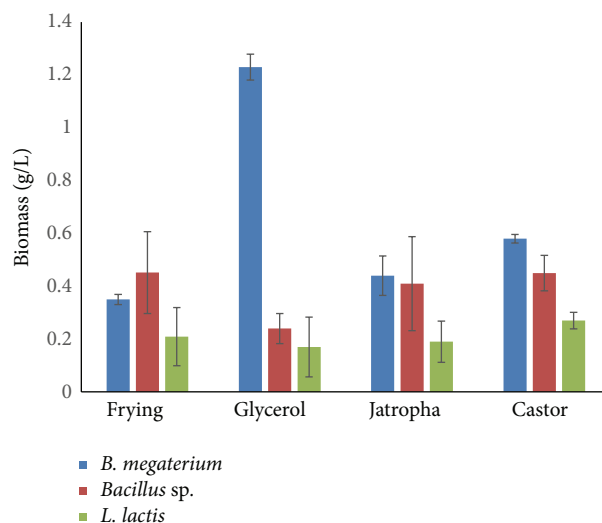
day by day) such as precipitated proteins, which can act as inhibitors affecting the fermentation process.

It is noteworthy to mention that the aim of this study is to find inexpensive carbon sources to overcome the performance obtained with other substrates to get PHA such as glucose, which is employed at industrial level. Thus, further studies are required to improve the performance with *B. megaterium*, as an alternative to produce PHA.

3.1.2. Evaluation with Bacteria-Oily Substrates and Bacteria-Glycerol Systems. The factorial arrangement established allowed the evaluation of the performance of the strains in media supplemented with oils (Jatropha, castor, and waste frying) and commercial glycerol. These tests allowed fast determination of the best bacteria-substrates combination, (i.e., production of biomass and PHA). Figure 5 illustrated comparatively biomass values obtained for each system. The best response was presented with the system *B. megaterium* glycerol, followed by *B. megaterium*-castor oil, *Bacillus* sp.-waste frying oil, and *Bacillus* sp.-castor oil.

PHA production was examined in the *B. megaterium*-glycerol systems, which showed the highest biomass production (1.23 ± 0.05 g/L), and *B. megaterium*-waste frying oil, which displayed lower bacterial growth (0.35 ± 0.02 g/L), but the carbon source is the cheaper of those tested in the present study. In *B. megaterium*-castor oil and *Bacillus* sp.-castor oil systems, there were bacterial growth and small amounts of polymer within their dry biomass extracts (<10 mg); thus, they were not subjected to GC/MS, since microbial growth is low and castor oil is heavily used in industries energy (e.g., the production of biodiesel) and in factories (e.g., production of paints); therefore the process for producing PHA should be better to those cases in order to compete with other industrial processes. Finally in systems with Jatropha oil, precipitation of the polymer was no visible.

In Figure 6, the spectrum (obtained by GC/MS) of the methyl ester derivative of commercial 3-hydroxybutyric acid
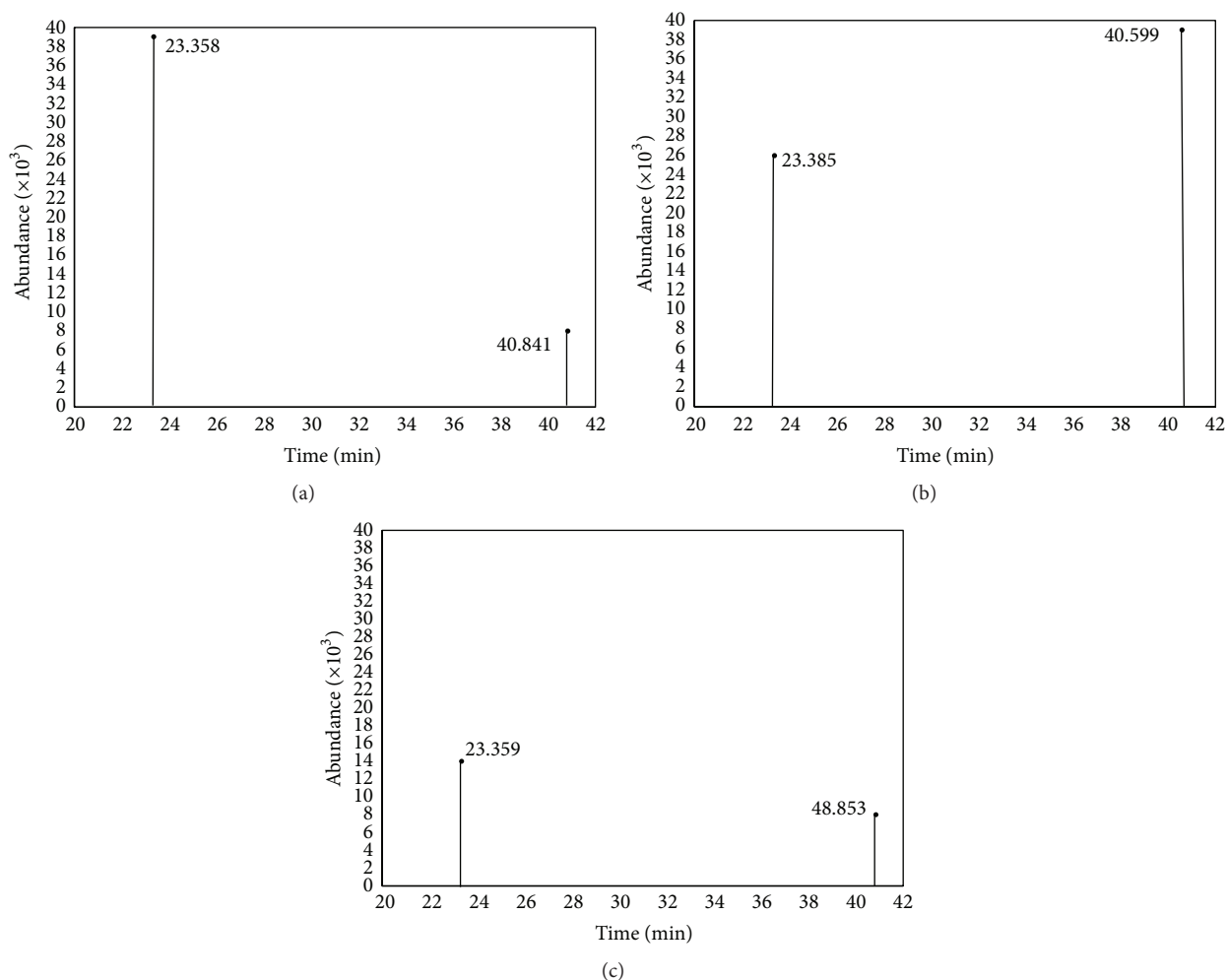
FIGURE 6: Mass spectrum (GC/MS) of the methyl ester derivative of (a) commercial 3-hydroxybutyric acid (PHB); (b) polymeric extract obtained with *B. megaterium* glycerol; and (c) the extract obtained with *B. megaterium* waste frying oil.

(PHB) is represented and the extract obtained in the system with *B. megaterium*-glycerol and *B. megaterium*-waste frying oil. The signal at 23.358 min corresponding to methylester methylether 3-hydroxybutyric acid (reference compound for quantification) and to the peak at 40.841 min is the internal standard (i.e., benzoic acid ester).

The best result obtained in the present study was *B. megaterium*-glycerol. In this system the biomass concentration at 48 h was 1.23 ± 0.05 g/L. This system was selected as the best bacteria-substrate system for the production of PHB in this study. However, it is important to mention that the presence of PHB in the system with waste frying oil is remarkable, but further improvements should be developed in additional studies.

Accordingly, and with the aim of reducing costs in the production of PHB, the following tests were made with the residual glycerol under the same operating conditions (i.e., substrate concentration, temperature, pH, and agitation) employed with the reagent grade glycerol. Thus, the quantification and optimization of the operating parameters with residual glycerol was established.

In these experiments, the fermentation time was reduced from 72 h, applied to systems with whey to 48 h, based on existing literature concerning the production of PHA in oil and glycerol matrices, reporting times less than or equal to 48 h. Thus, for example, Tian et al. [25], using *Pseudomonas mendocina* 0806 on substrates such as myristic and oleic acids characteristic oils tested in this research, found higher biomass accumulation and production of PHA at 48 h; Ng et al. [26] reported the same time for two variables, employing *Cupriavidus necator* H16 and Jatropha oil. Meanwhile, López-Cuellar et al. [38] reported increased production of PHB and biomass, after 40 h for *Wisteria eutropha* strain in canola oil; and Naranjo et al. [21] reported increased production of PHB, at 45 h, using a strain of *B. megaterium* and glycerol.

3.1.3. B. megaterium Residual Glycerol (RGSB) System. Glucose is the ultimate substrate, used in the PHA production at industrial level [2], which was considered as reference in this process. Data were obtained in our laboratory under the same operating conditions and are represented in Figure 7.
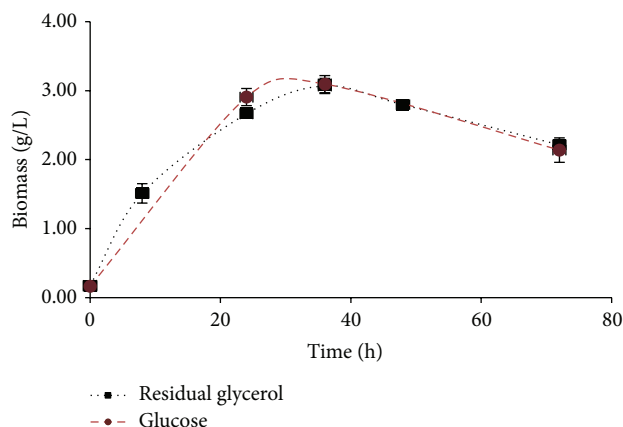
Figure 7: Growth curves of *B. megaterium* with residual glycerol and glucose.

B. megaterium displays a similar tendency of microbial growth in systems, reaching maximum values of 3.07 ± 0.11 g/L of biomass, in the medium with RGSB, and 3.096 ± 0.124 g/L, in the medium supplemented with glucose [23]. The enzymatic assembly allows the fast absorption of the substrates. Glycerol is a sugar alcohol that can be incorporated in the metabolic pathway of carbohydrates for the synthesis of PHA. Similarly, the bacterium contains enzymes able to degrade fatty acids in broths supplemented with oils.

Comparing the biomass obtained with GRG (1.23 ± 0.05 g/L;) at 48 h and that obtained with GRSB (2.80 ± 0.11 g/L;) under the same operational conditions the behavior in the first one was better. The microorganism exploits the different carbon sources present in the substrate, because possibly it is not completely pure. This behavior is according to Cavalheiro and coworkers [3], in systems with *C. necator*-pure glycerol (yield $Y_{x/s} = 0.37$ g$_{DW}$/g$_{gly}$) and *C. necator*-residual glycerol (yield $Y_{x/s} = 0.45$ g$_{DW}$/g$_{rgly}$).

The findings of this assessment and the ability of *B. megaterium* to accumulate PHA in GRG and its potential to produce PHA from RGSB were established, Thus, RGSB is very attractive for the production of biopolymers. In this sense, we preceded to optimize conditions for the production of PHB from RGSB.

3.2. Response Surface Designs

3.2.1. Dry Biomass Production and PHA.
The higher biomass production obtained with this system was 4.62 g/L, at 25°C (RGSB, 20 g/L, and pH, 8.0). In contrast, higher amount of PHA was 3.06 g/L at 25°C RGSB, 15 g/L, and pH 7.0, with a percentage of PHA of the dry biomass of 86.69% and yield $Y_{p/s}$ 204.1 mg/g.

Rising the temperature till 30°C triggers higher biomass production (3.69 g/L) at RGSB 15 g/L and pH 9.0, and the PHA production was 0.81 g/L at RGSB 15 g/L and pH 5.0, with a percentage of PHA from the dry biomass equivalent to 96.89% and yield $Y_{p/s}$ 54.0 mg/g.

With the increase of the temperature of the system to 35°C, the production of biomass was increased (4.46 g/L)

employing RGSB, 15 g/L, and pH 5.0. Meanwhile most PHA production (0.74 g/L) occurred while employing RGSB, 20 g/L, and pH 6.0 with a percentage of PHA of the dry biomass of 45.04% and yield $Y_{p/s}$ 36.8 mg/g.

Thus, higher temperatures affect negatively the bacterial growth and the accumulation of PHA.

3.2.2. Response Surfaces for Biomass Production.
In Figures 8–10, the system behavior (*B. megaterium*-RGSB) relative to the biomass produced under the conditions evaluated according to the substrate concentration (residual glycerol) and the pH is illustrated. Moreover, statistical models adjusted to the system estimated values that maximize biomass production (optimal) of each of the factors studied, for each temperature evaluated (25°C, 30°C, and 35°C).

In Figure 8, constructed for biomass at 25°C, a curvature surface is observed with a possible maximum concentration of biomass for 22 g/L and pH 9.0. Moreover, in conditions close to 8 g/L and pH 7.0, the region of lower concentration is observed for biomass. In the intermediate region up "apparent" biomass at pH values close to 15 g/L, pH 7.0 is observed. The contour plot (Figure 8(b)) displays the tendency of the system to the maximum biomass concentration points.

In Figure 9(a), the surface for the biomass at 30°C is displayed; a curvature occurs towards a possible biomass concentration (8 g/L, pH 9.0). At 8 g/L and pH 5.0 on the surface the lower concentration of biomass for the system was observed. The contour plot (Figure 9(b)) displays the tendency of the system to the maximum biomass concentration points.

In Figure 10(a), the surface for the biomass at 35°C is displayed, in which the tendency of the system to its maximum value is displayed in the selected region to system conditions around (15 g/L illustrated, pH 9.0). The contour plot (Figure 10(b)) shows the tendency of the system to the maximum biomass concentration.

3.2.3. Response Surfaces for PHA Production.
The surfaces obtained (Figures 11–13) display regions evaluated and behavior *B. megaterium* residual glycerol system, regarding PHA production, under the proposed conditions of operation.

In Figure 11(a), the concentration of PHA at 25°C displays the maximum concentration of PHA observed in the region of selected study values close to 15 g/L, pH 7.0. Moreover for values of 8 g/L, pH 9.0, the lowest concentration for PHA is observed. The contour plot (Figure 11) displays the region where the peak concentration for PHA, the conditions of pH, and substrate concentration are selected in the experimental design.

The concentration of PHA at 30°C (Figure 12(a)) displays a region of the maximum possible concentration of PHA in the system presented (15 g/L, pH 5.0); for values of 8 g/L, pH 9.0, the point is observed on the surface of lower concentration for PHA accumulation. The contour plot (Figure 12(b)) displays the tendency of the system to the maximum concentration PHA.

In Figure 13(a), the concentration of PHA at 35°C is illustrated. In this case, a saddle point occurs for values between 10 g/L and 15 g/L and pH 7.0 and pH 8.0, with
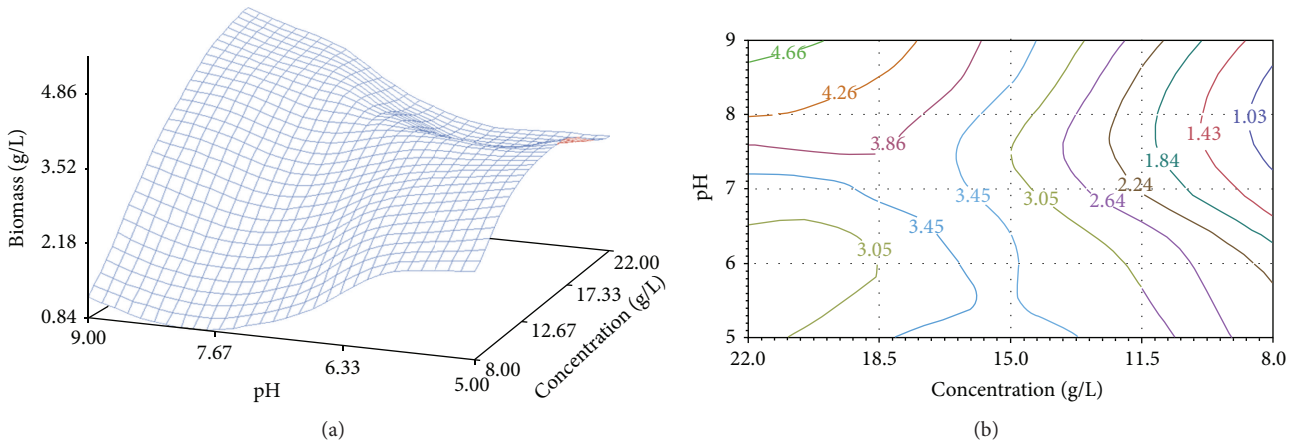
(a)

(b)

FIGURE 8: Biomass obtained in the *B. megaterium* residual glycerol system at 25°C. (a) Response surface for biomass. (b) Contour diagram for biomass in function of pH and substrate concentration.
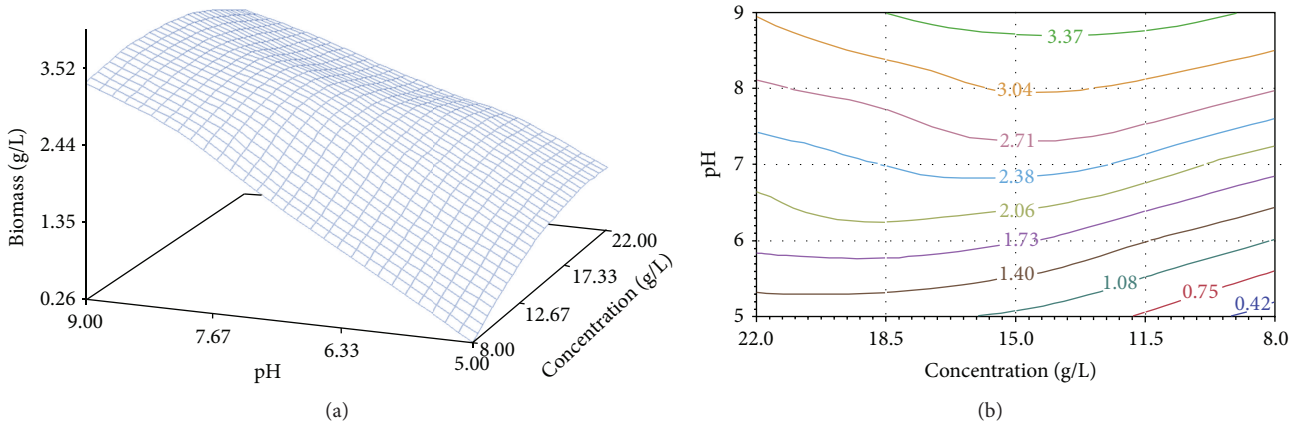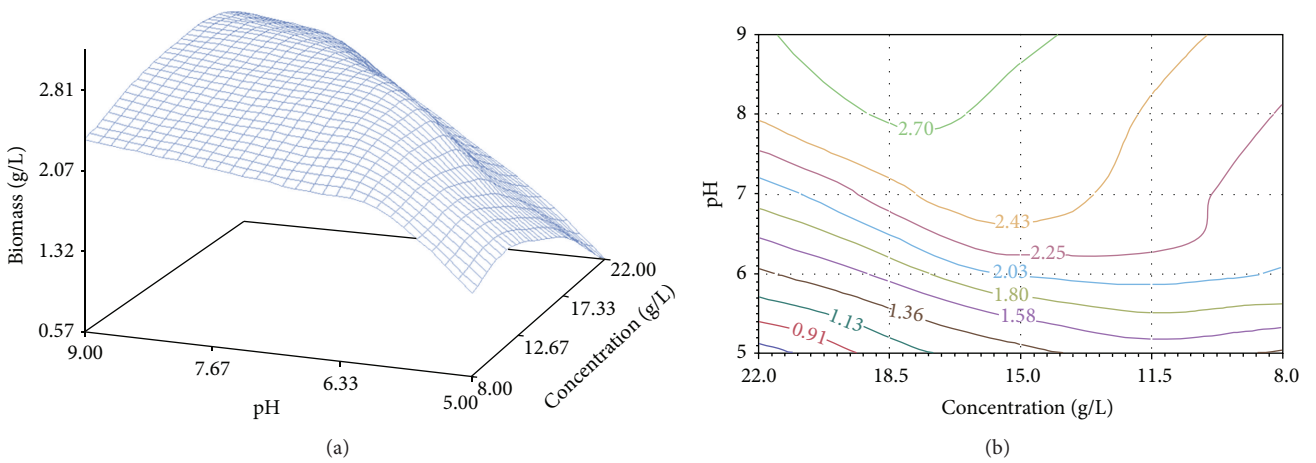

(a)

(b)

FIGURE 9: Biomass obtained in the *B. megaterium* residual glycerol system at 30°C. (a) Response surface for biomass. (b) Contour diagram for biomass in function of pH and substrate concentration.

(a)

(b)

FIGURE 10: Biomass obtained in the *B. megaterium* residual glycerol system at 35°C. (a) Response surface for biomass. (b) Contour diagram for biomass in function of pH and substrate concentration.

(a)

(b)
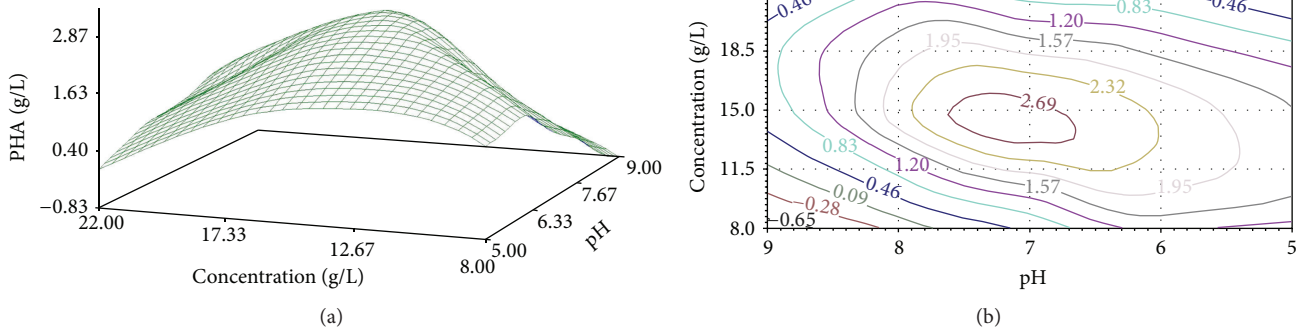
FIGURE 11: PHA production in *B. megaterium* residual glycerol system at 25°C. (a) Response surface obtained for PHA accumulation. (b) Contour plot for PHA production in function of substrate concentration and pH.
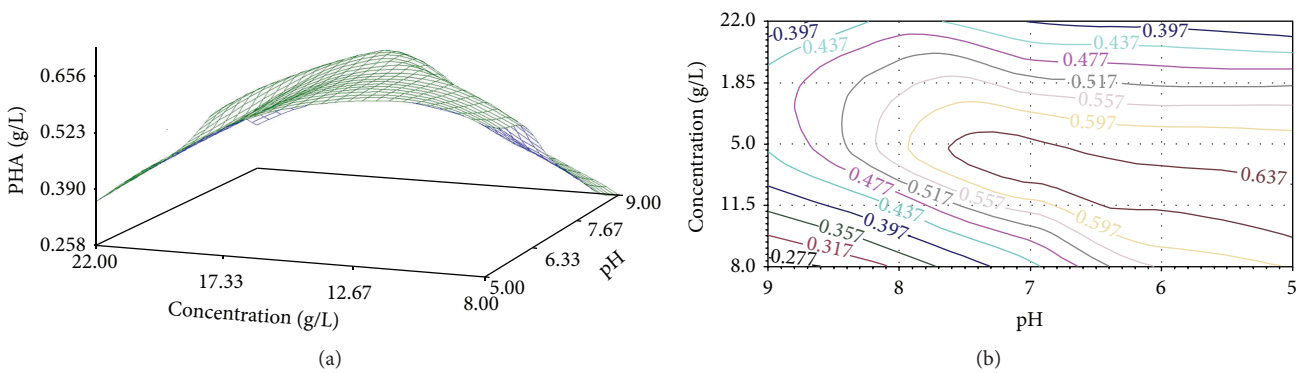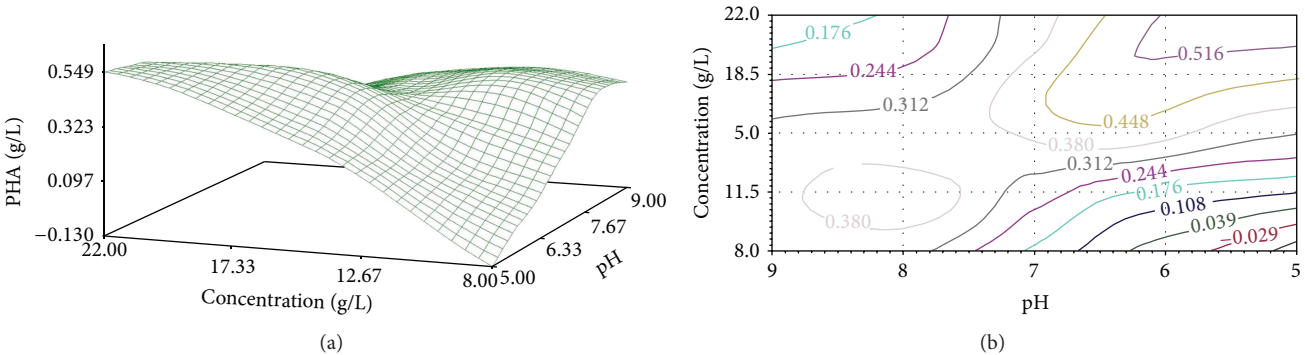

(a)

(b)

FIGURE 12: PHA production in *B. megaterium* residual glycerol system at 30°C. (a) Response surface obtained for PHA accumulation. (b) Contour plot for PHA production in function of substrate concentration and pH.

(a)

(b)

FIGURE 13: PHA production in *B. megaterium* residual glycerol system at 35°C. (a) Response surface obtained for PHA accumulation. (b) Contour plot for PHA production in function of substrate concentration and pH.

a maximum possible for values close to 22 g/L and pH 9.0. The contour diagram (Figure 13(b)) displays the tendency of the system to the maximum concentration of PHA.

3.2.4. Response Surface for Substrate Yield Global Product $Y_{p/s}$. Surfaces found (Figures 14–16) to yield PHA per unit substrate ($Y_{p/s}$) show the regions of interest for the target system.

In Figure 14(a), yield $Y_{p/s}$ at 25°C is displayed and a curvature is observed to the maximum concentration possible

$Y_{p/s}$, close to 15 g/L, pH 7.0. The extreme conditions (22 g/L, pH 5.0) and (8 g/L, pH 9.0) had the minimum system values for $Y_{p/s}$. The contour plot (Figure 14(b)) displays the tendency of the system to yield $Y_{p/s}$.

For yield $Y_{p/s}$ at 30°C (Figure 15(a)), it is clear that maximum performance is not in the study area proposed for optimization. However, it can be seen that for acid pH values and low substrate concentrations, the yield increases. The contour plot (Figure 15(b)) shows the tendency of the system to yield $Y_{p/s}$.
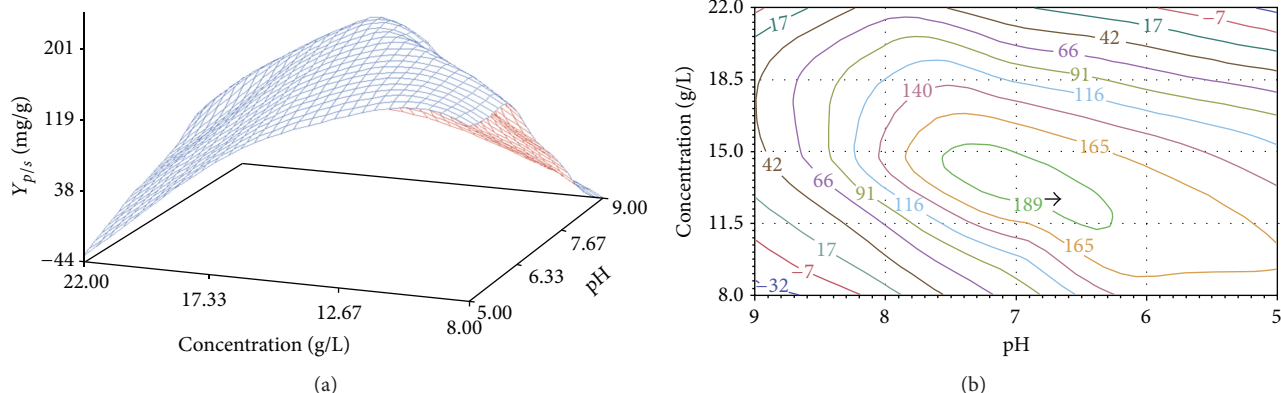
(a)

(b)

FIGURE 14: PHA yield per unit of substrate in *B. megaterium* residual glycerol system at 25°C. (a) Response surface obtained for $Y_{p/s}$. (b) Contour plot for the surface obtained in function of substrate concentration and pH evaluated.
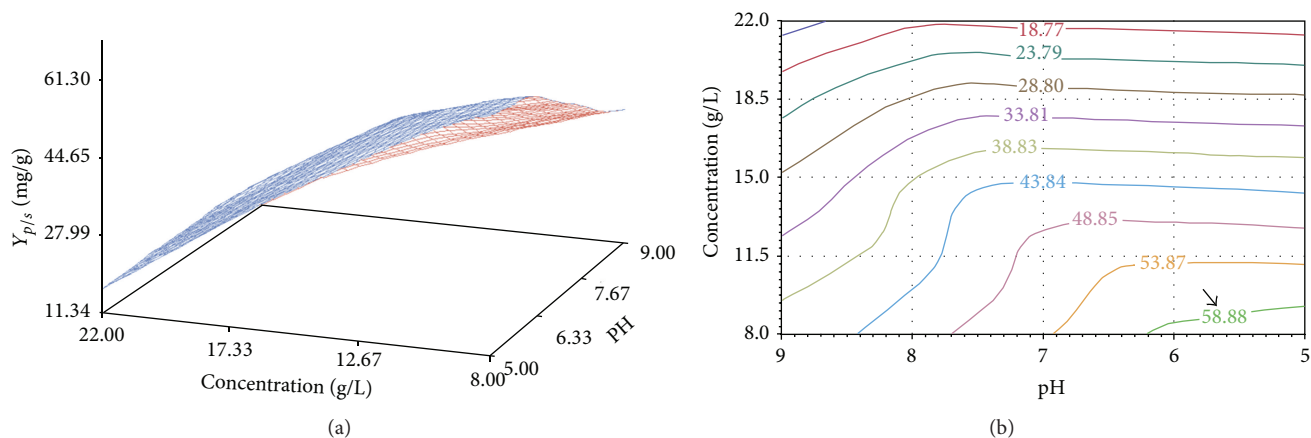

(a)

(b)

FIGURE 15: PHA yield per unit of substrate in *B. megaterium* residual glycerol system at 30°C. (a) Response surface obtained for $Y_{p/s}$. (b) Contour plot for the surface obtained in function of substrate concentration and pH evaluated.
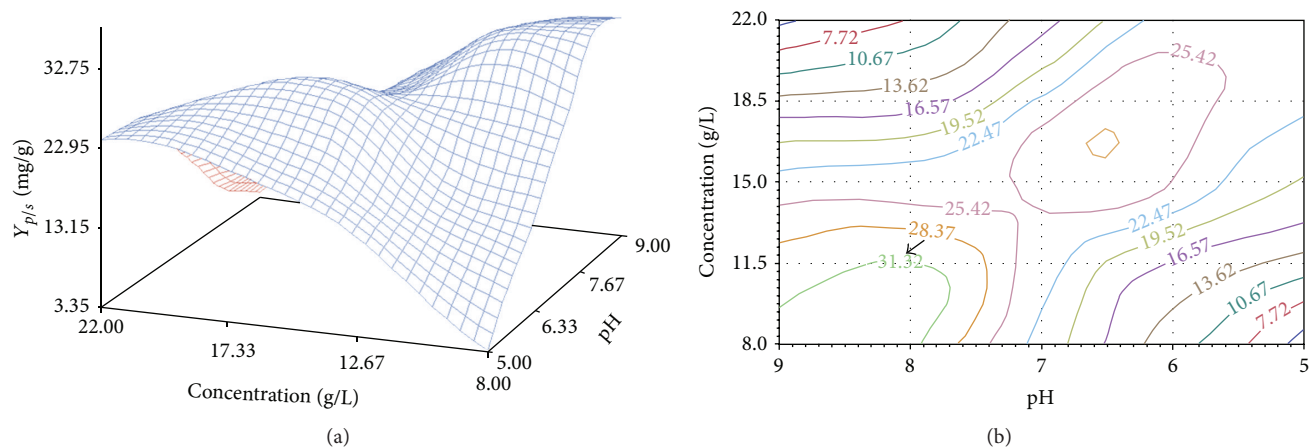

(a)

(b)

FIGURE 16: PHA yield per unit substrate in *B. megaterium* residual glycerol system at 35°C. (a) Response surface obtained for $Y_{p/s}$. (b) Contour plot for the surface obtained in function of substrate concentration and pH evaluated.

In Figure 16(a), yield $Y_{p/s}$ at 35°C is illustrated. The tendency of the system values is displayed to its maximum value close to 10 g/L and pH 8.0 values shown. The contour plot (Figure 16(b)) shows the tendency of the system to maximum yield $Y_{p/s}$.

4. Conclusions

All substrates tested (castor oil, frying oil, Jatropha oil, GRG, RGSB, and whey) were suitable for the growth of the strains *B. megaterium*, *Bacillus* sp., and *L. lactis*; however, the low biomass concentrations obtained for some systems are an obstacle for PHA production.

B. megaterium strain has the property of growing in sugar substrates, oily substrates, and glycerol; the latter has the advantage of producing PHB in amounts similar to those produced from sugar residues, under the operating conditions used in this experiment.

By cultivating native strain *B. megaterium* in liquid medium supplemented with GRG as a source of carbon, it is from a combined bacteria-substrate system very promising for the production of PHB, which allows using RGSB to 81.6% v/v to obtain 2.80 g/L PHB, a yield of $Y_{p/s}$ 186.8 mg/g in a process operated at 25°C, pH 7.0, and substrate concentration 15 g/L.

The optimal values for the system are given under operating conditions of RGSB 14.8 g/L, pH 6.7, and 25°C, with PHB production of 2.81 g/L and product yield $Y_{p/s}$ 189.9 mg/g.

The work area to optimize *B. megaterium*-RGSB system and obtain greater amounts of biopolymer (estimated in g/L) is at temperatures close to 25°C, residual glycerol concentrations close to 15 g/L, and pHs close to neutrality.

The use of *B. megaterium* residual glycerol system to PHB production is a doubly green process; it would solve the problem of biodiesel industry associated with the generation of residual glycerol and it would generate an added value, contributing to reduce pollution by petroleum-based plastics.

Conflict of Interests

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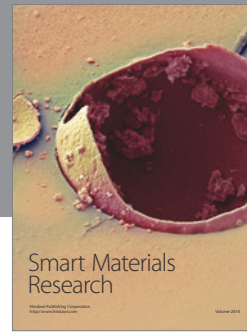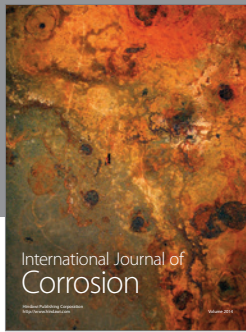
Acknowledgments

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