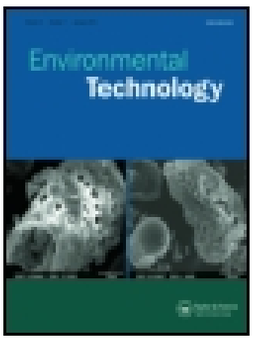

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Modeling the bioconversion of starch to P(HB-co-HV) optimized by experimental design using *Bacillus megaterium* BBST4 strain

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ABSTRACT

Poly(hydroxybutyrate-co-hydroxyvalerate) (P(HB-co-HV)) is a prominent biopolymer as a potential candidate for use in the biomedical area. Several *Bacillus* spp. strains show promising characteristics in the use of several carbon sources and are an interesting alternative for the production of P(HB-co-HV). Sewage from the agricultural and food processing industries can be used to obtain abundantly starch as a carbon source for PHA production. The aim of the present study was to optimize by response surface methodology and desirability, the production of PHA by a *Bacillus megaterium* strain using starch as the sole carbon source. Two optimal conditions were determined without sporulation and were used to perform new experiments to calibrate and validate a mechanistic model, developed to simulate the dynamics of PHA and biomass production. The developed model successfully represents the kinetics of the microorganism. Employing different characterization techniques, it was determined that the PHA produced by the strain is a copolymer composed of different HB:HV proportions. Using starch as the sole carbon source in a minimal salt medium, this work shows the first reports in the literature of: 1) a mathematical model for predicting growth kinetic and PHA production for *B. megaterium* strain and 2) a *Bacillus* spp. producing P(HB-co-HV) copolymer.

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Polyhydroxyalkanoate copolymer; starch; experimental design; modeling; yeast extract

Nomenclature

X	Total biomass concentration (g_{cell}/L)
R	Residual biomass concentration (g_{cell}/L)
P	Biopolymer concentration (g_{prod}/L)
S_1	Carbon source concentration (g_{sub}/L)
S_2	Nitrogen source concentration (g_{sub}/L)
t	Time (h)
μ	Specific growth rate (h^{-1})
μ_M	Maximum specific growth rate (h^{-1})
K_S	Saturation constant based on nitrogen concentration (g_{sub}/L)
k_1	Growth-associated product formation constant (g_{prod}/g_{cell})
k_2	Non-growth-associated product formation constant (g_{prod}/g_{cell}.h)
k_3	Growth-associated rate constant in carbon source consumption (g_{sub}/g_{cell})
k_4	Product-associated rate constant in carbon source consumption (Equation 6) (g_{sub}/g_{prod})
k_5	Non-growth-associated rate constant in carbon source consumption (g_{sub}/g_{cell}.h)
Y_{R/S_2}	Residual biomass yield based on nitrogen source (g_{cell}/g_{sub})
m_{S2}	Maintenance coefficient based on nitrogen source (g_{sub}/g_{cell}.h)
H	Hydrogen ion concentration (g_{H+}/L)
k_{ph1}	Growth-associated rate parameter in hydrogen ion concentration (L/g_{cell})
k_{ph2}	Growth-associated accumulation parameter in hydrogen ion concentration (L/g_{cell}.h)

1. Introduction

Polyhydroxyalkanoates (PHAs) are one of the main bio-based polymers considering current worldwide biopolymers production [1]. They are natural biodegradable polymers produced by bacteria in their cytoplasm as a carbon reserve [2] that would contribute to reduce the environmental impact generated by disposal of synthetic plastics [3]. Considering this characteristic and the high dependence that nowadays materials have from fossil fuels, PHAs became an attractive alternative to conventional petrochemical plastics due to their mechanical and thermal properties [4,5]. Furthermore, PHAs can be used in packaging, as well as pharmaceutical and medical applications due to their biocompatibility and slow hydrolytic degradation [6]. More common PHA, polyhydroxybutyrate (PHB) homopolymer, does not possess good material properties for the industry, because of its crystalline and brittle nature. On the other hand, PHA

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copolymers, such as poly(hydroxybutyrate-co-hydroxyvalerate) (P(HB-co-HV)), are more strong and flexible, and of greater industrial interest [7,8]. P(HB-co-HV) can be highlighted as an interesting candidate to be used in biomedical areas [9–11].

One of the main problems that the biopolymer industries face is the high cost of carbon sources for the fermentation process [12]. Even though there is a broad range of substrates that can be used as carbon sources for PHA production. In this context, the use of agricultural materials as substrates, commonly derived from renewable sources, such as starch, seems to be a promising alternative to substantially reduce the costs of substrate and process [13,14]. Starch is available abundantly from plant sources and wastewaters from agricultural and food processing industries, such as that obtainable from potato, corn, wheat and other starch-based waste streams. A significant part of these solid and liquid wastes is often thrown indiscriminately at landfills or even water sources, generating later health problems due to their decomposition [15], and are an attractive proposition for microbial processing in countries where there is a surplus of wheat, corn, potato, etc. [16]. Anyway, starch needs an extra step of hydrolysis [17–19] to be used as a substrate, since the microorganisms employed in major studies do not produce these enzymes natively, which increases the process costs. Bacterial strains co-producer of starch hydrolyzing enzyme and PHA are a good alternative to be employed.

Under certain cultivation conditions (defined media/nutritionally poor media), the strains require some substances/elements or precursors at particular concentrations to satisfy the nutritional requirements for their growth [20], which can be supplied by different nutritional supplements such as yeast extract (YE), among others. In this sense, the yeast waste from the brewing industry (yeast surplus), that represents a problem for this type of industry, can be an interesting and economical alternative [20] to be used as supplements for the production of factors and other precursors needed to stimulate the growth and production of PHA copolymers, such as P(HB-co-HV), produced by different strains of the genus *Bacillus*[21,22]. Moreover, *Bacillus* spp. strains exhibit some interesting characteristics such as low nutritional requirements, fast growth, osmotic tolerance and secretion of a large number of amylases and proteases [23], which make them a promising alternative to be considered in industrial applications. Unlike polymer obtained from Gram-negative bacteria, polymers produced by *Bacillus* spp. strains are free from endotoxins and can be used for biomedical applications with a reduction in purification costs. However, *Bacillus* spp. strains are spore-forming bacteria when an adverse environment is imposed. Sporulation takes place, generally, when the maximum concentration of PHA is reached; for this reason, it is important to determine the optimal growth conditions aiming to obtain high PHA production minimizing the sporulation process.

The response surface methodology (RSM) is a set of statistical and mathematical techniques for development, improvement and optimization of processes [24]. Also, it is a valuable tool to examine the interaction between factors and quantitatively represents the effects of the parameters used in the measurement of their responses [25,26]. Multiple factors and their interactions can also be effectively evaluated from the RSM [27,28]. To determine the best interactions and simultaneously optimize multiple functions, desirability equations defined by Derringer and Suich [29] are generally used.

Different variables influence the production of PHAs, such as the type of organism, the duration of fermentation, the growth rate, the nature and concentration of carbon source and the supplements added to the culture media [30]. Among the works found in the literature for optimization of PHA production processes, different authors have used variables such as starch in *Azotobacter chroococcum* and *Bacillus cereus* CFR06 [14,26], YE in *Bacillus megaterium* JK4h [31] and the growth time in *Thermus thermophilus* HB8 [32]. These variables have been used along with other independent variables, but after a comprehensive literature search, this work would be one of the first in which these three variables are used together to optimize the PHA production process.

Due to the fact that RSM provides an empirical model, it is highly recommended to complement this approach with mechanistic models in bio-based processes [33]. These deterministic models are based on dynamic mass balances for substrates, products and biomass that take into account biological process kinetics and their implementation can lead to a better understanding of the microorganisms' behavior through time [34]. Furthermore, bearing in mind that a similar behavior would be found for the flask and bioreactor modeling configurations [35], detailed kinetic information for basic cellular processes is a prerequisite to effectively describe the dynamics of a bioprocess [36]. For instance, Wang et al. [37] performed fluid dynamics assessment based on integration with microbial kinetics in order to scale-up industrial bioprocesses. Thus, the model can be used to predict the process development in different operating conditions, providing the temporary concentration profiles of the main components of the system. Among the applications of mechanistic models, the possibility to perform an extrapolation for fed-batch

fermentations adding terms of dilution, including the feeding of nutrients, can be highlighted [34,38], allowing future studies of nutrient feed strategies for polymer production enhancement [38]. In this sense, based on an exhaustive search in the open scientific literature, there are no kinetic models developed for growth and PHA production for *B. megaterium* strain employing starch as a sole carbon source in a minimal salt medium.

The aim of the present study was to: (i) optimize the production of PHA copolymer composed of HB and HV by a strain of *B. megaterium* using starch as the sole carbon source in the presence of YE and (ii) develop a dynamic mathematical model as a predictive tool of the process. The objective of the optimization was to achieve the most economically viable equation for this process to maximize the production of P(HB-co-HV) copolymer, minimizing: the growth time, the carbon source and the YE amounts employed and the possibilities of sporulation of the strain. Experimental design, based on RSM and desirability, was used for process optimization. Optimal conditions were experimentally verified and used to design the mathematical model, based on differential-algebraic equations (DAEs). Finally, the characterization of the PHA produced under the optimal conditions was performed using Fourier transform infrared spectroscopy (FTIR), DSC and ^{1}H-NMR.

2. Materials and methods

2.1. Microorganism and culture media

The PHA-producing bacterial strain used in the present work was isolated from sediments of the Bahía Blanca Estuary. According to biochemical tests and 16S rDNA analysis, the strain was identified as *B. megaterium* and denominated BBST4. The strain was stored at −72°C in Trypticase soy broth (Britania B0210206, Argentina) with 20% glycerol.

Culture media used in this study was salt solution (SS) containing NaCl, 2.5 g/L; K_2HPO_4, 2.5 g/L; KH_2PO_4, 2.5 g/L; $MgSO_4{\cdot}7H_2O$, 2.5 g/L; $MnSO_4{\cdot}4H_2O$, 0.05 g/L; $FeSO_4{\cdot}7H_2O$, 0.5 g/L amended with 1 mL of trace element solution, containing $ZnSO_4{\cdot}7H_2O$, 2.25 g/L; $CuSO_4{\cdot}5H_2O$, 1 g/L; $CaCl_2{\cdot}2H_2O$, 2 g/L; H_3BO_4, 0.20 g/L, with a final pH of 8, adjusted with NaOH 1 N solution. The SS was supplemented with starch (Anedra, Argentina) at different concentrations and 0.8 g/L of urea (Anedra, Argentina) (SSS) as sole carbon and nitrogen sources, respectively. SSS amended with 12 g/L ultrapure agar-agar (Merck, Germany) (ASSS) was used as a solid medium for growth. All media solutions were prepared with bi-distilled water. SS was autoclaved at 1 atm for 15 min. Starch, urea and trace element solution were sterilized by filtration through a membrane with 0.22 µm of pore (Merck-Millipore, Brazil) and added aseptically to the medium. Incubations were performed in a Gerhardt SW 20 (Denmark) thermostatic water bath shaker.

2.2. Shake flask cultivation

For the experiments, *B. megaterium* BBST4 from −72°C stock was recovered in Trypticase soy broth and incubated at 30°C in a shaker at 150 rpm for 24 h. Then, an aliquot was taken with a sterile loop and transfer on ASSS (with 1% starch and 0.08% urea) and the plates were incubated for 48 h at 30°C. One colony was picked and transferred to 100 mL flask with 25 mL of SSS (with 1% starch and 0.08% urea) and the flask was incubated at 30°C and 150 rpm for 48 h. The culture was harvested by centrifugation (ROLCO CM 4080, Argentina) for 15 min at 2000*g*, washed twice with saline solution (NaCl 0.85% w/v), and finally suspended in the same solution. Optical density of suspension was adjusted to an absorbance of 0.20 at 550 nm (OD550), corresponding to 10^8 colony forming units/mL (CFU/mL), using a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA). The resulting cell suspension was used as an inoculum for the experiments.

Sterile Erlenmeyer flasks of 250 mL were prepared with 100 mL of SS and the concentration of substrates and the growth times were determined through a central-composite design (CCD) defined to apply the RSM. Each flask was inoculated with 1 mL of the cell suspension, to obtain a final bacterial density of 10^6 CFU/mL. Experiments were designed to generate response surfaces to assess the effects on four dependent variables (Y): PHA accumulation (Y_1), cell growth (Y_2), starch consumption (Y_3) and pH variation (Y_4). Three independent variables (X) were employed in the design of the experiments: starch concentration (SC) used in the experimental culture medium (X_1), with the variation of the SC in relation to a constant nitrogen concentration; YE concentration (X_2), and final time of cell growth (Gt) in the sample (X_3).

Lower concentrations of YE compared with typically used in culture media were used, in order to assure the contribution of trace elements absent in the SSS medium that could assist in the growth and PHA production.

Different culture conditions were carried out according to a five-level central-composite circumscribed orthogonal blocking design (CCD). It follows a 2^3 factorial treatment with seven replicates at the central point plus six axial points.

Even when the strain and experimental conditions are the same, it should be considered that it is a biological process, so small changes either in the equipment or in the inoculums could produce slight variations in the results. Hence, to include these variations and in order to obtain more robust results, three blocks of experiments were performed. A total of 21 experimental runs were carried out and each experiment was assayed in triplicate in order to consider the internal experimental error.

2.3. Optimization variables

2.3.1. PHA accumulation (Y_1)

PHA extraction was determined at the end of each experiment according to the CCD and was performed using a modification of the chloroform extraction technique described by Manna et al. [39] and Valappil et al. [40]. Lyophilized cells from each sample were lysed by chemical digestion with a sodium hypochlorite solution (5.5% v/v in water) at 37°C for 1 h with constant shaking, centrifuged at 6000*g* and the obtained solid fraction was washed twice with distilled water, acetone and ethanol successively. Then, the solid was subjected to an extraction with hot chloroform for 15 min and filtered. PHA was recovered and purified from chloroform by precipitation with hexane, filtration and solvent evaporation. This method was used to obtain high-purity biopolymers [41]. PHA accumulation was referenced as PHA concentration (g/L). In order to complement the PHA extraction data of the CCD and to obtain a general PHA production curve tendency for each condition of the design, in all culture broths the PHA at several times (up to 50 h) was monitored using the Sudan black technique developed by our research group [42].

2.3.2. Cell growth (Y_2)

Experiments were carried out in a shaker at 30°C and 150 rpm. Cell growth was determined at the end of each experiment according to the CCD. The remaining volume from each flask was centrifuged at 2000*g* for 15 min to harvest the cells. The solid fraction was washed twice with distilled water and finally lyophilized (RIFICOR L-A-B3-C, with a WELCH 1402 vacuum pump). Subsequently, the lyophilized cell concentration (g/L) was determined in an analytical balance (Mettler AE 163, Mettler-Toledo Ltd, Leicester, UK). The resulting lyophilized biomass was used for PHA extraction as it is described above. In order to complement the cell growth data of the CCD and to obtain a general growth curve tendency for each condition of the design, in all culture broths the growth at several times (up to 50 h) was monitored by optical density at 550 nm.

2.3.3. Starch consumption (Y_3)

Starch content in the culture media was determined spectroscopically by measuring the absorption of the colored iodine–starch complex [43]. For this determination were employed 1 mL of sample with the addition of 0.1 mL of iodine solution (Britania, Argentina) and distilled water to bring the volume to 10 mL. Absorption was measured in a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA) at 550 nm [44]. Starch molecules formed a dark blue complex with iodine molecules, the absorption of this complex being proportional to the amount of starch present in the solution [45].

2.3.4. pH variation (Y_4)

The final pH was measured in each experiment to determine the pH variation (ΔpH) through the following equation:

$$\Delta pH = pH_o - pH_f \quad (1)$$

where pH_o was the initial pH, equal to 8 in all experiences, and pH_f was the final pH.

2.4. Sporulation

The percentage of sporulated cells was determined by the Schaeffer-Fulton staining method [46]. Slide films were made from samples and fixed by flaming three times. Then flooded with malachite green solution and heated to steaming three or four times within one-half minute. Washed and contrasted by applying a 0.5% of aqueous safranin solution. Finally, the films were washed, dried and examined in light microscopy with 100× oil-immersion objective.

2.5. Statistical analysis

The experimental data obtained from the CCD were analyzed by RSM and multivariate optimization using R software [47] with *rsm* and *desirability* packages, respectively. The steps followed were the coding of factors, the development of a central-composite design and finally the search of desirable conditions (more information is detailed in the Supplementary material).

2.6. Modeling PHA production by Bacillus megaterium BBST4

2.6.1. Model stoichiometry and kinetics

A combination of different aspects from models developed by other authors [34,38,48] was taken into account. The main purpose was to formulate an appropriate mechanistic model which helps to understand

the relationship among the principal state variables (biomass growth, PHA production, carbon source consumption and pH variation) and to provide the information about the behavior of the microbiological system. The fermentation process is represented by a DAE system.

Regarding the assumptions made by Patwardhan and Srivastava [38], total biomass (X) is expressed as the contribution of two components, residual biomass (R), which is the catalytically active component, and biopolymer concentration (P) considered as an inert component.

$$X = R + P \tag{2}$$

Since *B megaterium* strains are considered that produce PHA in high quantities principally when there is an excess of the carbon source, growth kinetics equation only considers a nitrogen source limitation [49].

$$\mu = \mu_M\left(\frac{S_2}{K_{S2} + S_2}\right) \tag{3}$$

Residual biomass can be considered as proteins, nucleic acids and other cell components of the microorganisms excluding the accumulation of intracellular polymer.

$$\frac{dR}{dt} = \mu R \tag{4}$$

The mass balance equations used for the polymer production together with the substrates sources are the ones presented by Mulchandani et al.[48] and Khanna and Srivastava [34] in their publications.

$$\frac{dP}{dt} = (\mu k_1 + k_2)R \tag{5}$$

$$\frac{dS_1}{dt} = -\left(k_3\frac{dR}{dt} + k_4\frac{dP}{dt} + k_5 R\right) \tag{6}$$

$$\frac{dS_2}{dt} = -\left(\frac{\mu}{Y_{R/S_2}} + m_{S2}\right)R \tag{7}$$

Finally, to represent the pH of the medium, two equations were included to obtain an efficient adjustment of the experimental data. The first one is a differential equation similar to that proposed by Faccin et al.[49], which represents the concentration of the hydrogen ions, and the second one is the decimal logarithm of the reciprocal hydrogen ion concentration.

$$\frac{dH}{dt} = (k_{ph1}.\mu + k_{ph2})X.H \tag{8}$$

$$pH = -\log_{10}(H) \tag{9}$$

2.6.2. Parameter estimation for proposed model

Parameters estimation was formulated as a dynamic optimization problem using gPROMS' [50] maximum-likelihood formulation. This kind of criteria attempts to determine the value of the model parameters that maximize the probability that model fits the values obtained from the experiments (observed data).

Mean error (ME), relative error (RE) and index of agreement (d) are determined in order to show the goodness of fit of the implemented model with the measured data. A better description of these model performance measures can be found in Appendix 1 (Supplementary material).

Addressing the verification of the model accuracy in order to adequately represent the experimental data, a validation of the model was carried out using an independent data set and evaluating the model performance by the ME and RE described before.

2.7. Characterization

PHAs obtained using optimum conditions were characterized with FTIR, DSC and H^1-NMR as follows.

2.7.1. FTIR spectroscopy

PHA was dissolved in chloroform and drops of this solution were placed on a NaCl window obtaining a thin film of the biopolymer by solvent evaporation. The spectrum was obtained on a Nicolet Nexus FTIR equipment (Thermo Scientific, USA) in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} employing 40 scans per sample.

Three crystallinity indices (CIs) proposed by different authors were determined using FTIR spectra of the PHA (for this determination, the PHA films were used with a certain time from preparation: 12 h or more time of solvent evaporation [51]). CIs were calculated as the ratio between the intensities of two bands as follows: CI_0: 1382 cm^{-1}/1185 cm^{-1}[52], CI_1: 1230 cm^{-1}/1453 cm^{-1}[51], CI_2: 1453 cm^{-1}/1185 cm^{-1}[53]. CI_0 was obtained by normalizing the absorbance at 1185 cm^{-1} to that of the 1380 cm^{-1} band [52]. C_1 and C_2 were obtained determining the ratio between peak heights, measured using OMNIC software.

2.7.2. Nuclear magnetic resonance (1H-NMR)

The molecular structure of the obtained PHA was determined by nuclear magnetic resonance (NMR). ^{1}H-NMR spectrum was recorded on a Bruker 300 MHz equipment using deuterochloroform (Aldrich) as a solvent. Approximately 10 mg of PHA in 1 mL of solvent was used for the analysis. PHA composition was obtained based on the

^{1}H-NMR spectrum by the ratio of the integrated signals of the hydrogens corresponding to methyl groups [52].

2.7.3. Thermal properties (differential scanning calorimetry)

Thermal properties of the extracted biopolymer were measured by differential scanning calorimetry (DSC) using a Perkin Elmer Pyris 1 calorimeter calibrated with indium and zinc as standards. For each sample (about 10 mg of PHA), a cycle of heating–cooling–heating in the temperature range of 30–200°C with a rate of 10°C/min. Thermal properties were calculated using the cooling (crystallization) and the second heating (melting).

3. Results and discussion

Among the production costs of PHA, approximately 50% corresponds to the carbon source used [54]. Since the price of glucose is about twice the price of hydrolyzed corn starch, the use of starch as the carbon source leads to a decrease in the production cost of about 25%, and allows to define the capacity as well as the potential of the strain studied to hydrolyze starch and its derivatives. Furthermore, the production of PHAs from starch as a carbon source is generally carried out using Gram-negative bacteria. It is known that Gram-negative bacteria are important producers of potentially harmful substances (endotoxins), so when considering some of the possible applications of PHAs, especially in the biomedical fields, the use of Gram-positive bacteria is more convenient [55].

3.1. Optimization variables

The implemented CCD scheme is shown in Table 1. Natural and encoded independent variables, the blocks and the number of run for each experiment are presented.

3.1.1. PHA accumulation (Y_1), cell growth (Y_2) and starch consumption (Y_3)

3.1.1.1. Influences of SC, YE and Gt. PHA production by *B. megaterium* BBST4 in all media studied suggests an efficient use of starch as the carbon source (Table 1). Maximum concentrations of extracted PHA were observed in experiences 1, 4, 12 and 14, with SC of 5 and 15 g/L, exceeding in all cases 0.4 g/L.

Based on growth curves of *B. megaterium* BBST4 (Figure 1), good biomass production between 10 and 25 h can be observed. Several authors have shown that after 18 h of growth generally appears to have an important diminution of SC in the medium [56–59]. This diminution seems to start in advance in *B. megaterium* BBST4 strain, which could be observed especially in experiences with SC below 10 g/L (Figure 2). For SC of 10 g/L, the main consumption of starch takes place before 25 h (Figure 2), while for higher SC used, starch degradation occurs at a relatively constant relationship. This last statement can be clearly observed with the higher SC (17 g/L). From the CCD, the strain appears to reach the stationary phase between 18 and 24 h in all culture conditions, except for SC = 10 g/L and YE = 0.05 g/L and SC = 15 g/L and YE = 0.08 g/L, where the exponential growth seems to be extended until at least 48 h. In addition,

Table 1. Experimental design (CCD) defined for RSM.

			Natural variables			Coded variables				
Exp.[a]	Order	Block	*SC* (g/L)	*YE* (g/L)	G_t (h)	X_1	X_2	X_3	PHA (g/L)	SD (±)
1	1	1	15	0.02	78	1	−1	1	0.510	0.017
2	2	1	10	0.05	48	0	0	0	0.210	0.007
3	3	1	15	0.08	18	1	1	−1	0.180	0.006
4	4	1	5	0.02	18	−1	−1	−1	0.409	0.006
5	5	1	10	0.05	48	0	0	0	0.282	0.005
6	6	1	10	0.05	48	0	0	0	0.249	0.004
7	7	1	5	0.08	78	−1	1	1	0.174	0.002
8	1	2	10	0.05	48	0	0	0	0.218	0.002
9	2	2	15	0.08	78	1	1	1	0.28	0.002
10	3	2	5	0.02	78	−1	−1	1	0.151	0.005
11	4	2	10	0.05	48	0	0	0	0.200	0.006
12	5	2	15	0.02	18	1	−1	−1	0.419	0.009
13	6	2	10	0.05	48	0	0	0	0.261	0.001
14	7	2	5	0.08	18	−1	1	−1	0.416	0.001
15	1	3	17	0.05	48	1.414	0	0	0.272	0.01
16	2	3	10	0.05	6	0	0	−1.414	0.062	0.003
17	3	3	10	0.0*	48	0	−1.414	0	0.284	0.01
18	4	3	3	0.05	48	−1.414	0	0	0.184	0.006
19	5	3	10	0.05	48	0	0	0	0.197	0.001
20	6	3	10	0.05	90	0	0	1.414	0.052	0.002
21	7	3	10	0.09	48	0	1.414	0	0.212	0.007

[a]Exp.: Experiment: each experiment was carried out in triplicate. *Control. C: carbon source, N: nitrogen source, SC: carbon:nitrogen rate, YE: yeast extract, Gt: growth time, SD: standard deviation.

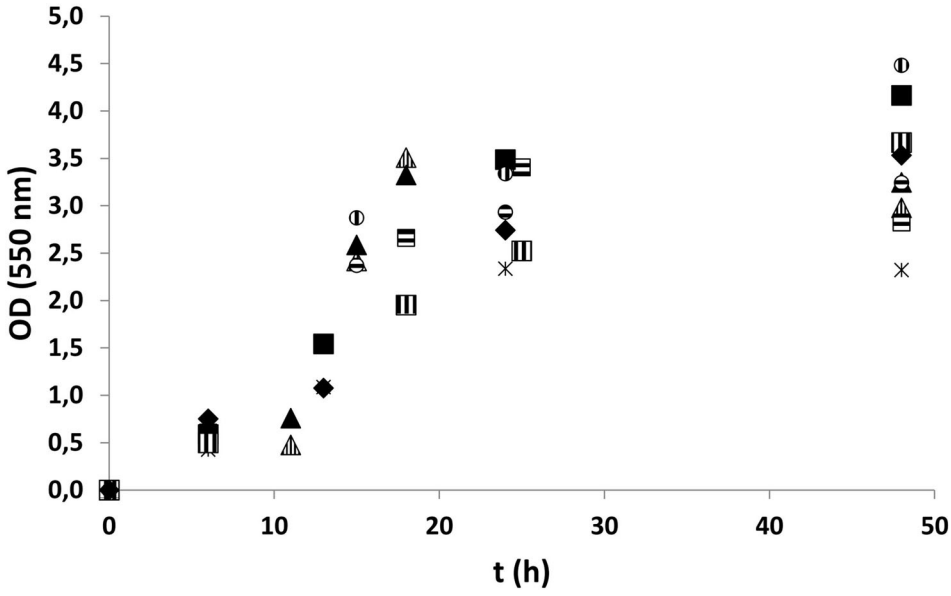

Figure 1. Cell growth as a function of time (G_t(h)) for CCD experiences. Symbols: (✳) SC = 3 g/L y YE = 0.05 g/L, (⟑) SC = 5 g/L y YE = 0.02 g/L, (▲) SC = 5 g/L y YE = 0.08 g/L, (〓) SC = 10 g/L y YE = 0 g/L, (■) SC = 10 g/L y YE = 0.05 g/L, (▥) SC = 10 g/L y YE = 0.1 g/L, (⊜) SC = 15 g/L y YE = 0.02 g/L, (◍) SC = 15 g/L y YE = 0.08 g/L, (◆) SC = 17 g/L y YE = 0.05 g/L.

experience with SC = 5 g/L seems to reach the stationary phase at an early period, close to 18 h**.** This time corresponds to the end of the slope of maximum consumption of starch and the maximum values of PHA production, observed for SC = 5 g/L of both concentrations of YE (Table 1). PHA production seems to decay in the end of longer growing periods for SC = 5 g/L, contrary to what is observed at SC greater than 10 g/L, which shows that PHA production continuously increasing after 48 h. However, with the higher SC (17 g/L) used in this work, despite continued growth observed after 48 h, production of PHA does not seem to be important and can be influenced by the excess of C and/or the lack of YE.

According to the model determined by RSM, the YE appears to have no impact on starch degradation by the strain studied (Table 3). However, the use of defined culture media may generate certain nutritional limitations for the PHA-producing strains under study, which means that certain substances/elements or

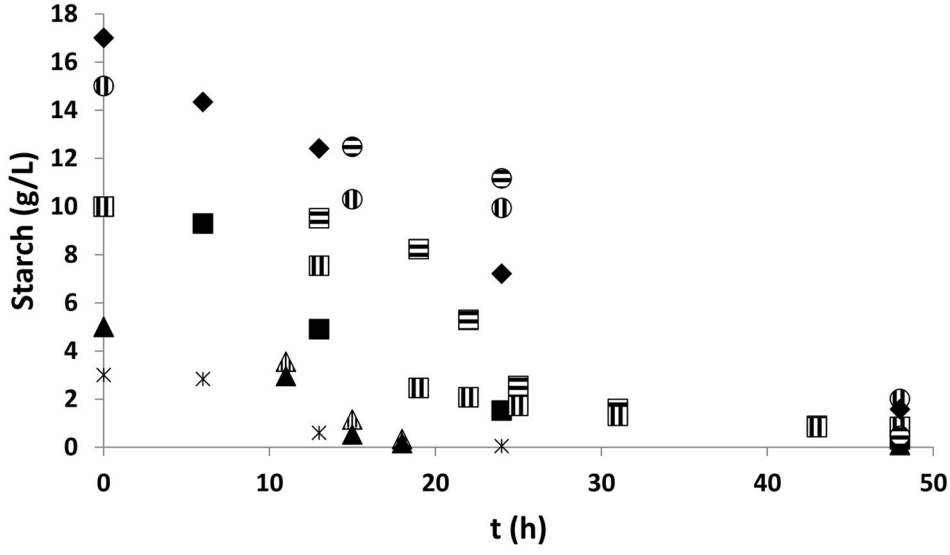

Figure 2. Starch consumption as a function of time (G_t(h)) for CCD experiences. Symbols: (✳) SC = 3 g/L y YE = 0.05 g/L, (⟑) SC = 5 g/L y YE = 0.02 g/L, (▲) SC = 5 g/L y YE = 0.08 g/L, (〓) SC = 10 g/L y YE = 0 g/L, (■) SC = 10 g/L y YE = 0.05 g/L, (▥) SC = 10 g/L y YE = 0.1 g/L, (⊜) SC = 15 g/L y YE = 0.02 g/L, (◍) SC = 15 g/L y YE = 0.08 g/L, (◆) SC = 17 g/L y YE = 0.05 g/L.

Table 2. PHAs production by bacteria using starch as a carbon source.

Strain	Culture[a]	Biomass (g/L)	PHA (%)	PHA (g/Lh)	Yields (g/g)[b] X/S	Yields (g/g)[b] P/S	Biopolymer	Work
Bacillus megaterium BBST4	D. SSS (5 g of S/L + 0.008 g of YE/L). 250 mL F (100 mL)	1.72	24	0.023	0.344	0.083	P(HB-co-HV)	This work
Bacillus sp. 112A	D. S (20 g/L). 500 mL F (100 mL)	1.85	50	0.026	0.093	0.046	PHB	[57]
Bacillus cereus CFR06	C. S (20 g/L + 0.5 g of YE/L). 250 mL F (50 mL)	1.0	48	0.007	0.050	0.024	PHB	[7]
Bacillus sp. CFR-67	RBH + WBH. 250 mL F (100 mL)	10.0	59	0.083	0.083	0.050	P(HB-co-HV)	[68]
	D. S. 250 mL F (100 mL)	2.5	60				PHB	
Halomonas boliviensis	C. SH (+MSG). 1 L F	9.2	56	0.160			PHB	[71]
	C. SH (+Xylose + MSG). 1 L F	8.3	45	0.120				
Halomonas boliviensis LC1	C. SH (+maltose + YE). F (310 mL)	1.2	56				PHB	[72]
	C. SH (+maltose + YE). 2 L B (2 L)	1.1	35					
Calidomonas taiwanensis	C. CS (15 g/L + 0.5 g/L of V + 0.1 g of YE/L). 250 mL F (30 mL)	2.8	67	0.058	0.181	0.121	P(HB-co-HV)	[73]
	C. MS 15 g/L + 0.5 g/L of V + 0.1 g of YE/L). 250 mL F (30 mL)	3.3	65	0.067	0.213	0.138		
	C. PS (15 g/L + 0.5 g/L of V + 0.1 g of YE/L). 250 mL F (30 mL)	2.6	55	0.045	0.168	0.092		
	C. WS (15 g/L + 0.5 g/L of V + 0.1 g of YE/L). 250 mL F (30 mL)	4.1	42	0.054	0.265	0.111		
Rhodobacter sphaeroides IFO12203	C. SaS (+Gluamy)1 L PBR (800 mL)	6.1	3	0.011		0.26	PHA	[61]
	C. SaS (+Amy + Glu)1 L PBR (800 mL)	3.8	2	0.014		0.18		
	C. SaS (+Amy + Gluamy)1 L PBR (800 mL)	6.0	3	0.019		0.30		
	C. SaS (+Pull + Gluamy)1 L PBR (800 mL)	4.6	3	0.012		0.25		
*Azotobacter chroococcum*23	D. S. 2.5 L FB (1 L)	54	46	0.350	0.059	0.043	PHB	[14]
	D. S (20 g/L). 500 mL F	1.17	74	0.015				
Cupriavidus sp. KKU38	D. CSH. B	5.97	41	0.025	0.299	0.125	PHB	[62]
Azotobacter chroococcum	D. S. 2.5 L B (1 L)	8.4	44	0.120	0.394	0.174	PHB	[74]
	D. S. 2.5 L FB	54	46	0.770				
Corynebacterium glutamicum	D. S. B	6.1	6	0.005	0.102	0.007	PHB	[55]

[a]D: defined media. C: complex media. YE: yeast extract. S: soluble starch. SSS: salt solution with starch. SH: starch hydrolysate. PS: potato starch. RBH: rice bran hydrolysate. WS: Wheat starch. WBH: Wheat bran hydrolysate. CSH: cassava starch hydrolysate. MSG: monosodium glutamate. CS: cassava starch. MS: corn starch. Amy: amylase. Gluamy: glucoamylase. Pull: pullulanase. Glu: glucose. V: valerate. SaS: Sago starch. F: flask, B: batch, FB: fed-batch, B: bioreactor. PBR: Photobioreactor.

[b]X/S: biomass yield coefficient; P/S: PHA yield coefficient.

precursors not present in the base culture medium are required for these strains to grow properly and produce other HA monomers other than HB. In this sense, *B. megaterium* BBST4 was able to produce a higher biomass, and PHA composed of HB and HV monomers using glycerol as a carbon source and growing in the presence of low concentrations of YE (0.005%) [22]. Thus, from the SC of 15 g/L, it was obtained 132% and 82% of increase in PHA using 0.02 g of YE/L (exp. 12 and 1, Table 1) in relation to the concentration of PHA obtained using 0.08 g of YE/L (exp. 3 and 9, Table 1), at 18 and 78 h of growth, respectively. Furthermore, for SC of 5 g/L with 0.08 g of YE/L (exp. 14 and 7, Table 1) it was obtained 2% and 15% more PHA in relation to the concentration of PHA obtained with 0.02 g of YE/L (exp. 4 and 10, Table 1), at 18 and 78 h of growth, respectively. These differences appear to show some YE influence with short hydrolysis times and high concentrations of starch and conversely, when the concentration of starch to be hydrolyzed is low. This observation is consistent with a positive influence of YE found for the production of α-amylase in *Bacillus* spp. strains at YE concentrations of 1 g/L [60] and 3 g/L [56]. On the other hand, with *Bacillus cereus* CFR06 strain and employing YE (0.5 g/L) and starch (20 g/L) in a growth medium for PHA production (Table 2), Halami [7] reported a higher percentage but lower yield and productivity of PHA than those obtained in the present work. Nevertheless, concentrations of YE used in such works are much higher than those used in the present work and, instead of being trace elements/growth factors, seem to be closer to act as direct carbon/nitrogen sources. On the contrary, in all cases the concentration of YE used in the present work is very low, which would generate a negligible term in the influence in nitrogen source concentrations, in the economic equations and therefore in production costs.

As in this study, different strains of *Bacillus* spp. and other PHA-producing strains (Table 2) produced the highest concentrations of PHA using SCs higher than 10 g/L and times longer than 30 h of culture [7,14,55,61,62]. On the other hand, generally PHA production is not so high at short times (e.g. 18 h) as those observed with *B. megaterium* BBST4 strain. PHA concentration obtained in this study was also higher than those obtained with *B. megaterium* JK4h strain, 0.342 g/L [31], using a less economical medium (3% glucose, 0.3% peptone and 0.075% YE), at growth times longer than

18 hours. It should also be noted that the yields reported in the literature for flask cultures were generally lower than those obtained in the present work (Table 2).

3.1.1.2. Productivities. Considering the need to reduce production costs, the maximum productivity of PHA obtained using 5 g/L of starch was similar to that obtained from 15 g/L (0.0231 and 0.0233 g/Lh, respectively), both at 18 h of growth. This would imply a 200% decrease in the cost of carbon source.

Productivities are similar to that obtained with various *Bacillus* spp. and higher than that obtained with different strains cultivated in batch culture using starch as the carbon source (Table 2), namely: *Bacillus cereus* CFR06 strain using 1% and 2% of soluble starch has produced 0.0056 and 0.0067 g/Lh, respectively [7], *Azotobacter chroococcum* H23 strain using 10% of starch has produced 0.0149 g/Lh [13] and *Corynebacterium glutamicum* ATCC13032 strain using 6% of starch has produced 0.0054 g/Lh [55]; all grown at 30°C and over 50 h of culture. Nonetheless, PHA and biomass yield coefficients determined in this study (for exp. 14) were higher in almost all cases compared with batch cultures presented in Table 2 using different strains and starch at many forms as a carbon source.

3.1.2. pH variation (Y4)

Experiments carried out with an SC = 5 g/L and YE = 0.08 g/L indicate high starch consumption at pH near 8, where in the pH of the medium ranged from 8 to a minimum of 7.4 at 18 h of growth, showing a nearly complete starch consumption before 20 h, with a marked slope of starch consumption before 10 h.

Variation of pH determined in relation to the initial pH, for experiences defined in the CCD, is shown in Figure 3. pH variation appears to be proportional to the initial concentration of starch and experimental time.

3.2. Sporulation

The peak of biopolymer accumulation occurs just before sporulation, being the PHA degraded during this process. This last would make the development of any bioprocess of PHAs production economically unfeasible [63]. However, the number of cells that showed sporulation was relatively low (<5%) and in most experiences was not observed, including the ones with longer growth times. In experiences with the lower SC (3 g/L), only 5–8% of sporulated cells after 48 h of culture was observed. From these results, it can be said that conditions used for the cultures did not stimulate the sporulation. On the other hand, *B. megaterium* BBST4 strain has the ability to grow long periods of time without presenting sporulation in a minimal salt synthetic medium using starch as the sole carbon source (SSS), with and without the addition of YE.

3.3. Statistical analysis

3.3.1. RSM determinations

Figure 4 shows response surfaces for optimal relationships between PHA production (Y_1) and factors (X_i) based on the

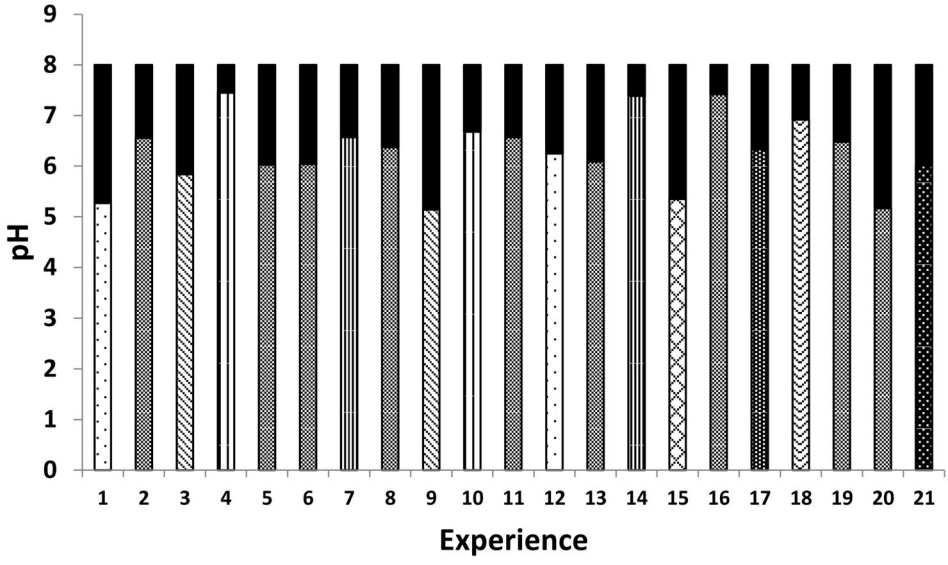

Figure 3. pH variation as a function of growth time (G_t(h)) for CCD experiences. Experiences: 1 and 12, SC = 15 g/L y YE = 0.02 g/L; 2,5,6,8,11,13,16,19 and 20, SC = 10 g/L y YE = 0.05 g/L; 3 and 9, SC = 15 g/L y YE = 0.08 g/L; 4 and 10, SC = 5 g/L y YE = 0.02 g/L; 7 and 14, SC = 5 g/L y YE = 0.08 g/L; 15, SC = 17 g/L y YE = 0.05 g/L; 17, SC = 10 g/L y YE = 0 g/L; 18, SC = 3 g/L y YE = 0.05 g/L; 21, SC = 10 g/L y YE = 0.1 g/L.

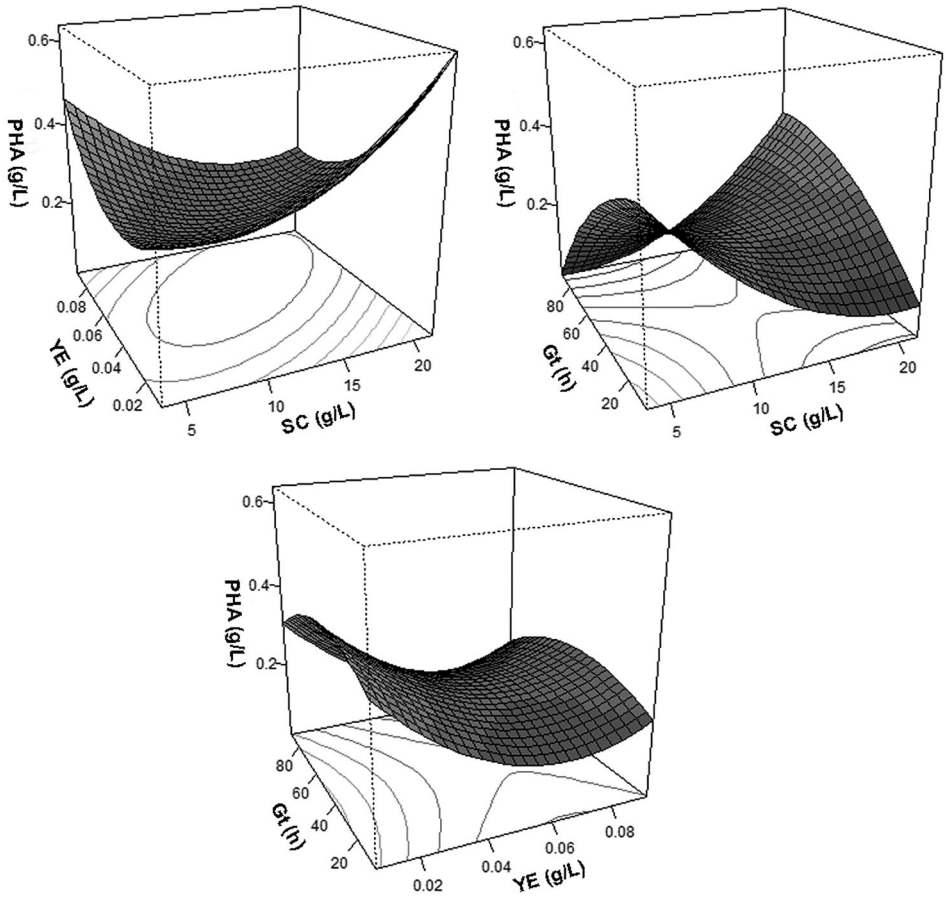

Figure 4. Response surfaces for PHA production (g/L) as a function of the analyzed independent variables (SC, YE and Gt).

experimental results determined for the experiences defined in the CCD (Table 1). RSM and desirability were used to define statistically the optimal culture conditions that minimize growth time maximizing the production of PHA. Its application defines the effect of independent variables alone or in combination, on the processes studied. In addition, in order to analyze the effects of independent variables, from RSM it is possible to generate a mathematical model (Table 3) that describes the relationship of the variables [27]. This last could not be possible using other classical methods for optimization (such optimization with simple variables) because it does not represent the full effects of process variables and ignores the interactions between them [64].

As noted in the models for *B. megaterium* JK4h strain [31], with the YE variable (*YE*) exception, the independent variables SC and Gt showed a highly significant effect on the response variables. On the other hand, all the independent variables seem to have high incidence on PHA production and only growth time seems to have incidence on biomass production (Table 3).

Adjusted R^2 determined for almost all models showed values near to 0.9, indicating a good fit considering that variables were not transformed, which generally improves those values.

3.3.2. Desirability

In any industrial process maximization of productivity, defined as the maximum profit obtained in the shortest possible time, is the goal. Following this idea and based on CCD-RSM determinations, desirable conditions of the predictor variables or factors were obtained for maximum PHA production at the minimum possible

Table 3. Equations determined by RSM for response variables

Equation determined by RSM	R^2
$Y_1 = PHA = 0.2476 + 0.0303X_1 - 0.0449X_2 - 0.0270X_3 - 0.0623X_1X_2 + 0.0864X_1X_3 + 0.0486X_1^2 + 0.0585X_2^2 - 0.0370X_3^2$	0.876
$Y_2 = Biomass = 1.9841 + 0.1687 - 0.3985X_3^2$	0.743
$Y_3 = Starch = 0.8576 + 1.9923X_1 - 2.8833X_3 - 2.6502X_1X_3 + 1.9783X_3^2$	0.858
$Y_4 = pHvariation = 1.7176 + 0.6509X_1 + 0.5377X_3$	0.878

Note: X_1: SC ratio; X_2: Yeast extract; X_3: growth time.

time. For this purpose, the *desirability* package for R software was used.

Desirable conditions determined for the applied RSM (Table A1, Supplementary Material) proved to be SC = 15 g/L and YE = 0.02 g/L at 28 h of culture as an optimal general condition and SC = 5 g/L and YE = 0.08 g/L at 18 h of culture as an optimal minimum condition that correspond to the first and third highest PHA production obtained in the CCD experiments, respectively (Table 1). These two conditions were called DCA and DCB, respectively.

3.3.3. Validation for the best experimental conditions

The validation experiences using the desirable conditions were denoted DCAVal and DCBVal for DCA and DCB conditions, respectively. These experiences were tested at five culture growth times (Figure A1, Supplementary Material). PHA concentrations predicted by desirability (Y_1) were in agreement with the PHA concentrations determined in validation experiences (PHA_v, Table A1, Supplementary Material).

3.4. Modeling, parameter estimation and model validation

Based on the desirability analysis, experimental data of both optimal conditions obtained were employed for the modeling of growth and PHA production. Data include DCA and DCB experiences, along with an independent set of experiences (DCAVal and DCBVal) used for model validations. A parameter estimation problem with six differential equations and three algebraic equations was solved. As Table 4 shows, the optimization problem for parameter estimation was similar for both cases since metrics of the model such as total CPU time, iteration number and final objective function values reflect a comparable complexity associated to the parameter association process.

Table 5 shows optimal values of the estimated parameters for the proposed model using DCA and DCB experiments. Parameters highlighted with an asterisk (*) were assumed to be the same ones employed by Faccin et al.[49].

The obtained results are in concordance with the physical phenomena explained by the mentioned authors [34,38,48,49]. Carbon source consumption is attributed to residual biomass growth and biopolymer formation as Equation (6) shows [38]. Biopolymer production represented by Equation (5) considers the possibility of PHA synthesis in growth and stationary phases [48]. The negative value of parameter k_2 allows to represent the microorganism mechanisms in which the biopolymer can be also consumed as a carbon source under eventual stressful conditions [34,48,49].

Table 4. Metrics for parameters estimation problem.

	DCA	DCB
Differential equations	6	6
Algebraic equations	3	3
Initial objective function	486.8	969.0
Final objective function	16.9	7.8
Iterations	24	26
Total CPU time (s)	1.5	1.9
X^{2a}	22.4	24.9
Weighted residual	17.4	18.5

[a]Good fit: Weighted residual less than chi-squared value (X^2).

Experimental data of DCA and DCAVal experiences and the simulated profiles of the main variables obtained after parameter estimation for the proposed model are shown in Figure 5.

Based on performance indexes presented in Table A2 (Supplementary Material), the model represents appropriately the studied system. The lower errors associated with carbon source (S_1) in comparison to polymer production (P) can be explained by the presence of more parameters in Equation (6) than in Equation (5). This fact would allow a better maximization of the model adjustment with Equation (6).

It is worth mentioning that any kind of error introduced by one of these variables affects the other one in an expectable way as Mulchandani et al.[48] asserted in their work, concluding that the residual biomass (R) can be considered as the catalytically active biomass.

The proposed model was validated using an independent set of experimental data (DCAVal) as it is shown in Figure 5. In comparison with the data set used for parameter estimation (DCA), it can be pointed out that *REs* turn out to be slightly higher for all the variables involved in DCAVal (Table A3, Supplementary Material). However, the proposed mechanistic model represents properly the behavior of the microorganisms under the studied conditions.

Table 5. Optimal parameter set for PHA production model for *Bacillus megaterium* BBST4.

	DCA	DCB
Parameter	Optimal value	
μ_M	1.996	3.100*
K_{S2}	7.002	5.384
k_1	0.222	0.189
k_2	-8.39×10^{-5}	-1.49×10^{-3}
k_3	0.9894*	0.9894*
k_4	22.9601	5.983
k_5	4.92×10^{-3}	0.010
$Y_{R/S2}$	2.895	2.798
m_{S2}	1.73×10^{-6}*	1.73×10^{-6}*
k_{ph1}	2.2873*	1.398
k_{ph2}	8.07×10^{-4}	1.95×10^{-3}

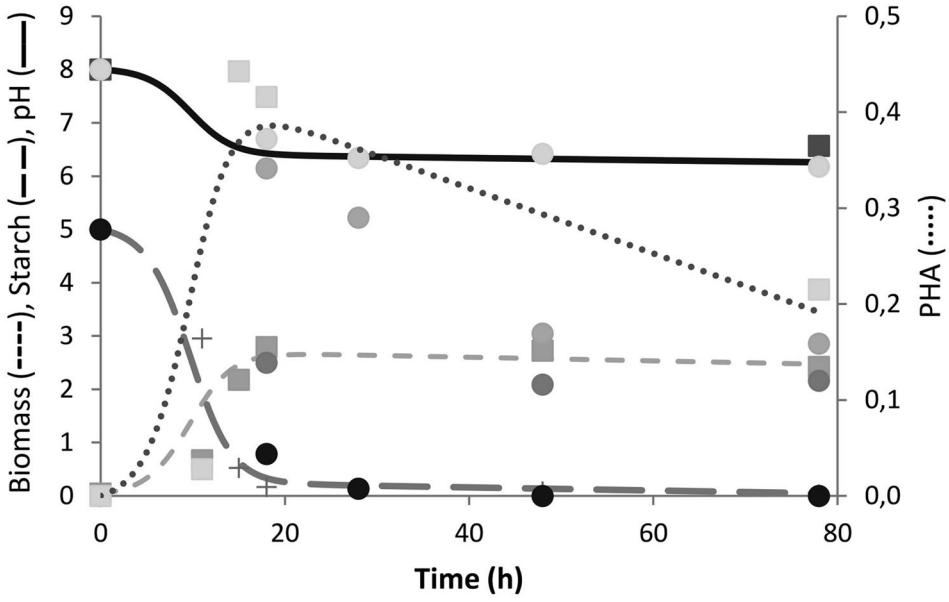

Figure 5. Experimental data and simulated profile for experiences DCA (■) and DCAVal (●) as a function of time (G_t(h)) for *Bacillus megaterium* BBST4 strain. (·····) PHA, (----) Biomass, (— —) Starch and (——) pH: Simulated profile for variables state.

Figure 6 shows the experimental data and the proposed model corresponding to DCB and DCBVal. As well as in the previous case, it can be noticed that the model successfully achieved the representation of the trend of each measured variable (Table A4, Supplementary Material). Furthermore, the most important REs associated with PHA (*P*) were not higher than 33%. This last, together with the *MEs* of the state variables which are close to zero, indicates a satisfactory parameter estimation of the model with the data set used for this purpose (DCB).

The validation process for the model based on DCB data was performed using a different data set (DCBVal) from the one used to estimate the parameters of the kinetic model as it was done for the previous case using DCA and DCAVal. The goodness of fit above 0.9 (Table A5, Supplementary Material) shows an appropriate fitting of the validation experiments.

The parameters estimated in the optimization problem shown in Table 5 are in good agreement with the results presented by Faccin et al.[49]. For instance, the case of the negative value of parameter k_2,

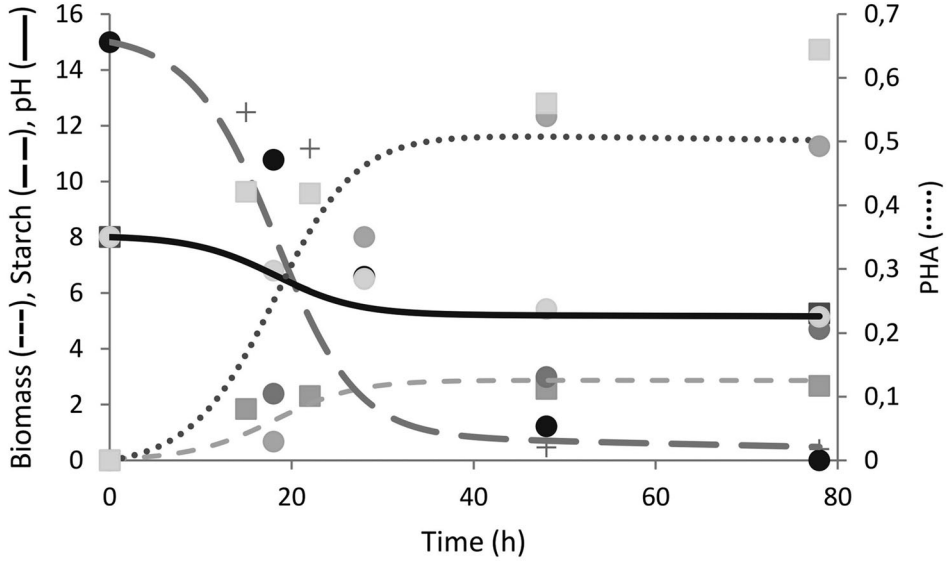

Figure 6. Experimental data and simulated profile for experiences DCB (■) and DCBVal (●) as a function of time (G_t(h)) for *Bacillus megaterium* BBST4 strain. (·····) PHA, (----) Biomass, (— —) Starch and (——) pH: Simulated profile for variables state.

showing the scenario in which the microorganisms can also consume PHA as a carbon source under specific conditions. Another aspect related with the former state is the fact that all the estimated parameters, except for $Y_{R/S2}$, adopted values considered by the standard deviation of the parameters reported in the literature [34,38,48,49]. The particular exception of parameter $Y_{R/S2}$ could be due to the fact that is highly dependent on the residual biomass:nitrogen source ratio employed in each experiment.

Furthermore, it can be noticed that the proposed mechanistic model (Equations (2–7)) after parameter estimation adequately represents the experimental data as it is shown in Figures 5 and 6. Therefore, this mathematical model effectively describes the microorganism kinetics; consequently, it can be employed for the future study of the optimal bioreactor strategies [36]. In addition, it also can be used to analyze PHA synthesis under different substrates and acidic initial conditions.

3.5. PHA characterization

3.5.1. Fourier transform infrared spectroscopy

FTIR spectrum of the biopolymer produced by the *B. megaterium* BBST4 strain shows intense absorption bands around 1730 cm^{-1}, 1380 cm^{-1} and 1280 cm^{-1} corresponding to the carbonyl group, CH bond of CH_3 group and carboxyl group, respectively, indicated that the produced biopolymer is a PHA [7,57,62]. Based on FTIR spectra, three crystallinity indexes (CIs), defined as the absorbance ratio between two FTIR reference bands, were determined. Although the crystallinity index is not the absolute degree of crystallinity, this index was useful to evaluate the crystallinity differences between PHAs [51].

Xu et al.[51] have shown that the typically reference band at 1382 cm^{-1}[52] can be used only for fast crystallizing PHAs, but is not suitable for copolymers containing monomers of short and medium chain length. Therefore, they suggested a new crystallinity index (CI_1). Subsequently, Luo et al. [53] suggested another index where the band at 1453 cm^{-1} is considered as a reference for measuring the degree of crystallinity (CI_2). In this work, CIs proposed by the three different authors were calculated and they are shown in Table 6.

CIs of PHA produced by *B. megaterium* BBST4 were compared with CIs of different PHAs, including a commercial PHB.

As can be seen, all CIs are lower than the corresponding for a commercial PHB, while the CIs for commercial PHB were similar to those determined for PHB homopolymer by Bloembergen et al.[52], Xu et al. [51] and Luo et al.[53]. Compared with CIs of pure PHB and copolymers, CI values for PHA produced by *B. megaterium* BBST4 are similar to the copolymer obtained by Bloembergen et al.[52] (Table 6). Based on these values, PHA produced by *B. megaterium* BBST4 must include other monomers different of HB in the PHA structure.

Table 6. Crystallinity indexes (CIs) determined for commercial PHB and PHAs produced by means of *Bacillus megaterium* BBST4 using FTIR.

Sample	CI_0	CI_1	CI_2
PHB*	0.99	2.51	0.83
PHA	0.95	1.70	0.51
P(HB-co-6%HV) [39]	0.96	–	–
P(HB-co-HHx) [38]	–	1.17	–
P(HB-co-mclHA) [40]	–	–	0.31

Notes: PHB: commercial PHB, PHA: PHA produced by *B. megaterium* BBST4 using starch as a carbon source, CI_0: Crystallinity index defined by Bloembergen et al. [52], CI_1: Crystallinity index defined by Xu et al. [51], C_2: Crystallinity defined by Luo et al. [53]. *CIs for PHB similar to standard PHB obtained by different authors [51–53].

3.5.2. Differential scanning calorimetry

Thermograms of the PHA produced by *B. megaterium* in the presence of YE (CDB sample) and PHB produced by the same strain using glucose as the sole carbon source in the absence of YE [65] are shown in Figure 7. Thermal properties of the obtained PHA show a melting curve with two peaks: one at 135.18°C and the second one at 155.85°C. Melting point value of PHA produced by *B. megaterium* was lower than values reported in the literature for pure PHB, 173–176°C [66] and is also lower than T_m obtained for pure PHB produced by the same strain using glucose as the sole carbon source (T_m = 170.6°C, [65]). Obtained T_m was similar to that of a PHA produced with the recombinant *Ralstonia eutropha* strain with 93.4 mol% of HB and 6.6 mol% of HA with 6–12 carbons [67]. Percentage of crystallinity of obtained biopolymer ($X\%_{PHA}$) was 56.6% and was calculated based on the melting enthalpy of a perfect PHB crystal (149.37 J/g, [65]). This $X\%_{PHA}$ showed to be lower than that determined for the PHB produced with the same strain using glucose as the sole carbon source ($X\%_{PHB}$ = 61.5, [65]). These data are relevant because a decrease in the melting temperature increases processability without degradation of the biopolymer. In addition, a decrease in the degree of crystallinity increases the degradation rate, since amorphous regions are degraded more rapidly than crystalline ones [40].

3.5.3. Nuclear magnetic resonance

^{1}H-NMR spectrum for the PHA produced by the *B. megaterium* BBST4 strain is shown in Figure 8. Doublets located among 1.25 and 1.29 ppm and 2.29 and 2.63 ppm, and the multiplet signal among 5 and 5.3 ppm, belonging to the methyl (CH_3), methylene

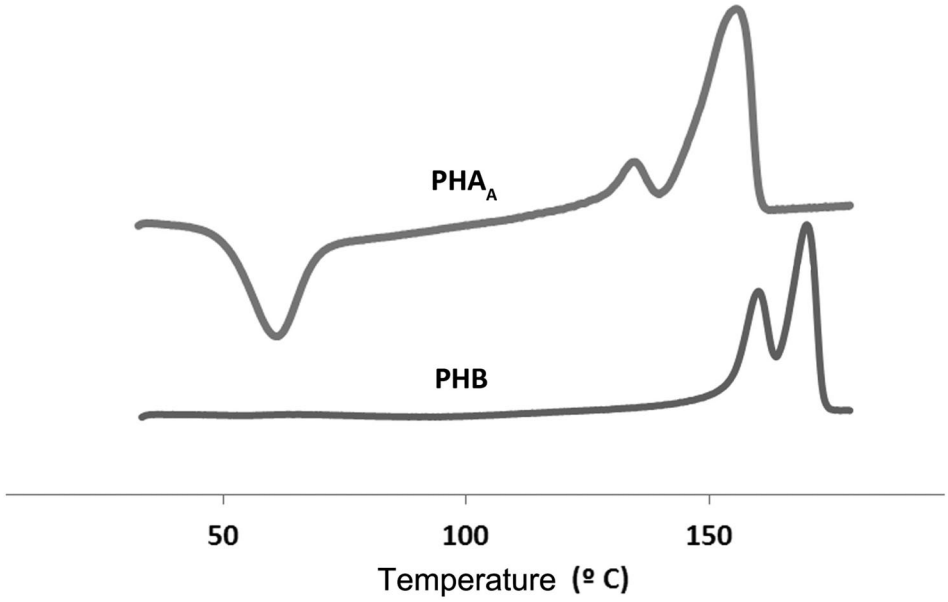

Figure 7. Thermograms corresponding to the second heating of the PHAs produced by the *B. megaterium* BBST4 strain. PHA_A: PHA produced using starch in presence of YE; PHB: PHB produced by the same strain using glucose in the absence of YE.

(CH_2) and methyne (CH) groups, respectively, and typically correspond to hydroxybutyrate (HB) [55]. Moreover, peaks at 0.90 and 1.6 ppm can be observed in the spectrum, and they indicate the presence of CH_3 and CH_2, respectively, of HV lateral chain in the biopolymer [18]. Polymer composition was calculated from the area ratio of absorption peaks in the methyl groups (1.27 for CH_3(4) and 0.90 for CH_3(9), from the structure shown in Figure 8) from ^{1}H-NMR and based on these characterization results, the biopolymer produced by *B. megaterium* BBST4 strain was a copolymer composed of HB and different fractions of HV (approximately 6.2 and 9.8 mol % for DCA and DCB samples, respectively). Apparently, *B. megaterium* BBST4 can increase HV production when the SC/YE ratio decreases. A similar condition was found in *Bacillus* spp. CFR-67 employing a complex medium with starch as the main carbon source and different concentrations of bran hydrolysates, where the strain produced HV fractions between 5 and 10 mol% [68]. Moreover, the presence of YE in the culture

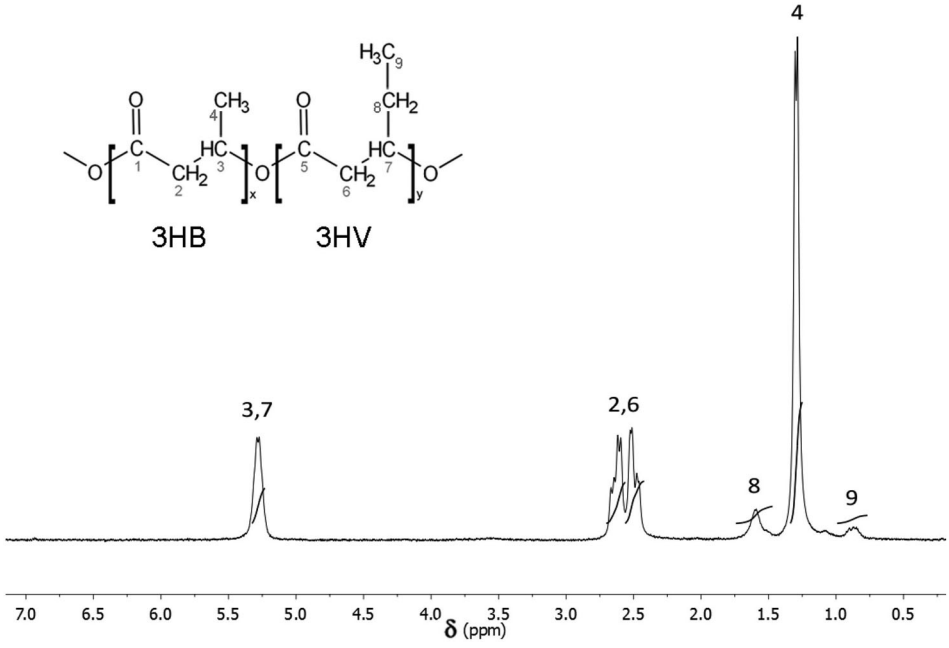

Figure 8. ^{1}H-NMR spectrum for the PHA produced by *Bacillus megaterium* BBST4 using starch as a sole carbon source.

medium appears to affect the ability to produce copolymers of P(HB-co-HV), favoring the input of precursors for the synthesis of the HV monomer. This can be inferred from the fact that the same strain can produce P(HB-co-HV) with similar characteristics using glycerol as the sole carbon source in the presence of low YE concentrations [22]. On the other hand, using glycerol or glucose as the sole carbon source and in the absence of YE, *B. megaterium* BBST4 only produces PHB homopolymer [69]. In the same way, Reddy et al. [21] was able to obtain copolymers of P(HB-co-HV) (with low HV concentrations) using another strain of *Bacillus* (*Bacillus* sp. 88D) and glycerol or glucose as the only carbon source in the presence of low concentrations of Ye (0.004 g/L), and Povolo et al. [70] achieved an increase in the HV production by the *Hydrogenophaga pseudoflava* DSM1034 strain with the addition of YE to the culture media containing lactose or sucrose as the sole carbon source.

4. Conclusions

PHA production by *B. megaterium* BBST4 was investigated through RSM and desirability, obtaining two optimal conditions without sporulation (SC = 15 g/L and YE = 0.02 g/L at 28 h of culture as an optimal general condition and SC = 5 g/L and YE = 0.08 g/L at 18 h of culture as an optimal minimum condition). These optimal conditions were taken into account to perform new experiments employed to calibrate and validate a mechanistic model for PHA and biomass production. Based on an exhaustive search in the open scientific literature, this is the first mathematical model to predict the kinetic of growth and PHA production for *B. megaterium* strain employing starch as the sole carbon source in a minimal salt medium. It was found that the model successfully represents the kinetics of the microorganism; therefore, it can be used as a predictive tool for further bioprocess optimization, such as the development of adequately feeding strategies in fed-batch bioreactors. In addition, this mathematical model has promising perspectives for industrial applications where the main purpose is the enhancement of PHA production. Finally, the biopolymer produced in optimized conditions was characterized and the results allowed determining that the PHA produced by the strain is a copolymer composed of HB and HV. The synthesis of HV is probably favored by the presence of precursors obtained by the strain from the YE present in the medium. This type of copolymer has advantageous properties as a material for tissue engineering, and is suitable for use as a substrate for implants and cell cultures which gives it a high added value. To our best knowledge, this is the first report of a *Bacillus* spp. with the ability to produce P(HB-co-HV) copolymer employing starch as the sole carbon source in the presence of YE in a minimal salt medium. Based on this ability, *B. megaterium* BBST4 can be used to degrade and give added value to many cheap starch-based wastes derived from agricultural and food processing industries. This development and the possibility of co-obtain PHA and α-amylase in the same process are the next steps of our investigations.

Geolocation information

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Disclosure Statement

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