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Modeling and Experimental Evaluation of Poly(3-hydroxybutyrate) Production in *Bacillus megaterium*

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Topic: Integration of life sciences & engineering.

Abstract

The aim of this research was the optimization of poly(3-hydroxybutyrate) – P(3HB) – production obtained in submerged cultures of *Bacillus megaterium* in a mineral medium, using sucrose as carbon source and the nitrogen as the limiting substrate. A bench-scale bioreactor was used in order to obtain experimental data for phenomenological modeling of the biopolymer production, microbial growth, and substrate consumption. From an experimental design, which was carried out in shaker at 30°C and 160 rpm, it was evaluated the best initial sucrose concentration and carbon-to-nitrogen ratio in order to maximize the biomass accumulation and biopolymer production. It was observed the P(3HB) accumulation in bacteria without the need of nitrogen limitation and a strong correlation between accumulated P(3HB) and pH. Bench-scale bioreactor experiments were carried out and showed the importance of a control pH strategy. The proposed model was implemented in the EMSO process simulator and its parameters were estimated using this tool and the experimental data from the bioreactor. The simulation results showed good agreement with the experimental data.

1 Introduction

Plastics have been regarded as ideal materials for the production of various consumer products because of their durability and inherent resistance to degradation. However, these same qualities are sources of environmental and waste management problems. These problems have created much interest in the development of biopolymer. In addition, biopolymers can be obtained from agriculture or from biotechnological processes and are therefore, in principle, available from renewable resources (Luengo et al., 2003; Reddy et al., 2003).

Polyhydroxyalkanoates (PHAs) biopolymers are polyesters synthesized by numerous bacteria and are accumulated on inclusion bodies in the cytoplasm of the cells as energy storage. They are produced from renewable carbon sources and, generally, with limiting nutrient; and their main characteristic is the biodegradability (Lee, 1996b; Madison and Huisman, 1999). Poly(3-hydroxybutyrate) (P(3HB)) is the most characterized PHA, partially crystalline polymer, and with material properties similar to polypropylene (Lee, 1996a; Sudesh et al., 2000).

The aim of this research was the optimization of poly(3-hydroxybutyrate) – P(3HB) – production obtained in submerged cultures of *Bacillus megaterium* in a mineral medium, using sucrose as carbon source and the nitrogen as the limiting substrate.

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2 Materials and methods

2.1 Microorganism and culture medium: The bacterium used in this study was *Bacillus megaterium*, DSM 32^T. The mineral medium is the same used in Wang and Lee (1997).

2.2 Design of experiments: Shaker experiments were carried out, according to a central composite design with three repetitions in the central point, in order to evaluate the best initial sucrose concentration (S) and carbon-to-nitrogen ratio (C:N) for increasing cell concentration and accumulated polymer. In Table 1, it was shown the concentration of sucrose and source of nitrogen (SN) to give the desired carbon-to-nitrogen ratio. Batch fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of culture medium with initial pH = 7. The flasks were inoculated and maintained at 30°C and 160 rpm for the requested time. Samples were collected in 4, 8, 12, 16, 20 and 24 hours.

Table 1: Design of experiments

EXP	X1 (S)	X2 (C:N)	S (g·L^{-1})	C:N	SN (g·L^{-1})
1	1	1	44	22	2.00
2	1	-1	44	8	5.50
3	-1	1	16	22	0.73
4	-1	-1	16	8	2.00
5	0	$-\sqrt{2}$	30	5	6.00
6	0	$\sqrt{2}$	30	25	1.20
7	$-\sqrt{2}$	0	10	15	0.67
8	$\sqrt{2}$	0	50	15	3.33
9a	0	0	30	15	2.00
9b	0	0	30	15	2.00
9c	0	0	30	15	2.00

2.3 Bench-scale bioreactor: For larger scale production, a 5 L bioreactor was used containing 4 L of the culture medium. The initial substrate concentration (16 g·L^{-1} of sucrose and 2 g·L^{-1} of ammonium sulphate) was chosen according to the optimal point of the surface response curve from the design of experiments.

Bench-scale bioreactor experiments were carried out with dissolved oxygen and pH control. Cultivations parameters were as follows: pH, 7; temperature, 30°C; aeration, 4 L/min; stirred speed, 200-700 rpm. The dissolved oxygen (pO_2) was controlled changing the stirred speed in order to keep the value of dissolved oxygen above 40%. Bench-scale bioreactor experiments without pH control, but keeping the other conditions unchanged, were also carried out.

2.4 Analytical procedures: Total biomass was evaluated by dry weight, from 40-10 mL of culture broth. The cell suspension was centrifuged at 3500 rpm for 20 min at 4°C, washed with distilled water, transferred to pre-weighed vials and dried in an oven at 80°C till constant weight. P(3HB) amount was determined by propanolysis of monomers according to the method of Riis and Mai (1988). Residual biomass was calculated total biomass less biopolymer amount, once biopolymer was accumulated into the cell. The sucrose was analyzed according to Dubois et al. (1956) and by HPLC using a Rezex-RHM (300 mm x 7.8 mm) column at 80°C and RI detector. Nitrogen was determined by the phenol-hypochlorite reaction according to Weatherburn (1967).

2.5 Modeling: Different mathematical models for microbial growth, biopolymer production and substrate consumption were analyzed (Khanna and Srivastava, 2005; Raje and Srivastava, 1998). A new model which is a improved version of the Khanna-Srivastava model presenting a lower number of parameters. This model is shown in Table 2. All models were implemented in the EMSO process simulator (Soares and Secchi, 2003) and their parameters were estimated using this tool and the experimental data from the bioreactor.

Table 2: Equation of the modified model

Residual Biomass(X_R):	$\frac{dX_R}{dt} = (\mu - k_d) \cdot X_R$
Specific grow rate (μ):	$\mu = \mu_m \left(\frac{S}{K_{SS} + S} \right) \cdot \left(\frac{N}{K_{NS} + N} \right)$
Biopolymer (P):	$\frac{dP}{dt} = (k_1 \cdot \mu + k_2) \cdot X_R$
Sucrose(S):	$\frac{dS}{dt} = -(\alpha \cdot \mu + \gamma) \cdot X_R$
Nitrogen (N):	$\frac{dN}{dt} = -\frac{\mu}{Y_{RN}} \cdot X_R$

3. Results and discussion

3.1 *Design experiments*

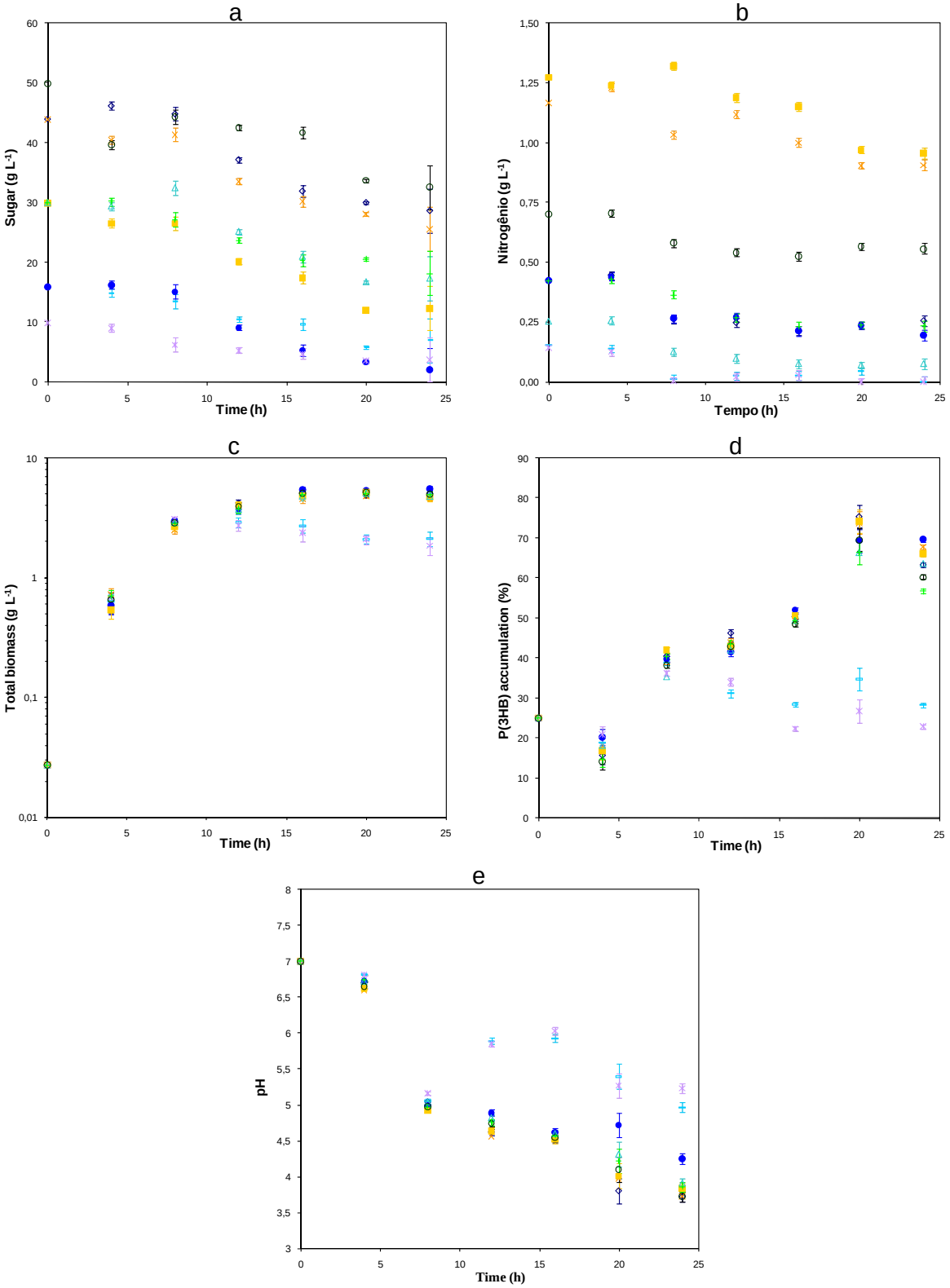


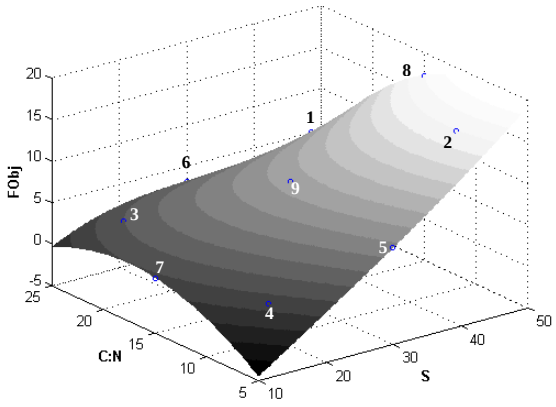
Figure 1: Time evolution of sugar concentration (a), nitrogen concentration (b), total biomass concentration (c), polymer accumulation (d) and pH (e) throughout the culture.

In the experiments, P(3HB) was accumulated in the cell about 75% at 20 hours of culture. In most of the cases, there was no limitation in the sucrose or nitrogen (Figure 1a and 1b) but a decreasing in P(3HB) accumulation after 20 h could be seen (Figure 1c) and no more increasing in the biomass could be detected (Figure 1d). The pH decreased with the time (Figure 1e) and it was observed a strong correlation between accumulated P(3HB) and pH of the medium.

It is observed that *B. megaterium* shows different behavior from bacteria commonly used on biopolymer production, like *R. eutropha*, since it is able to produce large amounts of P(3HB) without nitrogen limitation. Similar results are found in Omar et al. (2001) and Maccol et al. (1996).

The results of the design of experiments were analyzed using surface response for biomass, polymer accumulated, and residual sucrose. With the fitted equations from this surfaces it was constructed an objective function to determine the best condition to maximize the biomass accumulation and biopolymer production and also to minimize the residual sucrose.

This objective function is shown in equation (1) and also in Figure 2 with weight w = 0.2. The optimal point was found to be close to the experiment number 4 (S = 16 g·L^{-1} e C:N = 8), which was then chosen to carry out the experiments in bench-scale bioreactor.



$$F_{Obj}(S,C:N) = (1-w)\cdot S_R(S,C:N) - w\cdot\left(\frac{P(S,C:N)\cdot X_T(S,C:N)}{100}\right) \quad (1)$$

Figure 2: Plot of the objective function and the equation that represents this function.

3.2 *Bench-scale bioreactor*

The experiments without pH control (SCPH) and with pH control (CCPH) were done in duplicate in the bench-scale bioreactor. The results (medium values of duplicates) are shown in Figure 3. The SCPH experiment was finished earlier than the CCPH, after 12 h, because the dissolved oxygen came back to 100% (Figure 3e).

Comparing the results from bench-scale bioreactor with those from shaker we could observe that the polymer accumulation is much lower in the bioreactor, even without pH control, which is the condition corresponding to the shaker process. This decrease in the P(3HB) accumulation compared with shaker was also observed in Omar et al. (2001).

In Figure 3a, it can be observed that total biomass (Xt) and residual biomass (Xr) were lower in SCPH than in CCPH experiments, although the P(3HB) amount was similar. Therefore, P(3HB) accumulation (%) into the cell in SCPH experiments was higher than the CCPH (Figure 3b). As in SCPH experiments there was no limitation either in sucrose or in nitrogen, the cells growing interruption could be attributed to the final pH was very low. These results show that: i) the use of a pH based control strategy is very important and ii) maintaining pH = 7 during the whole process, as in CCPH experiments, is not the most adequate control strategy.

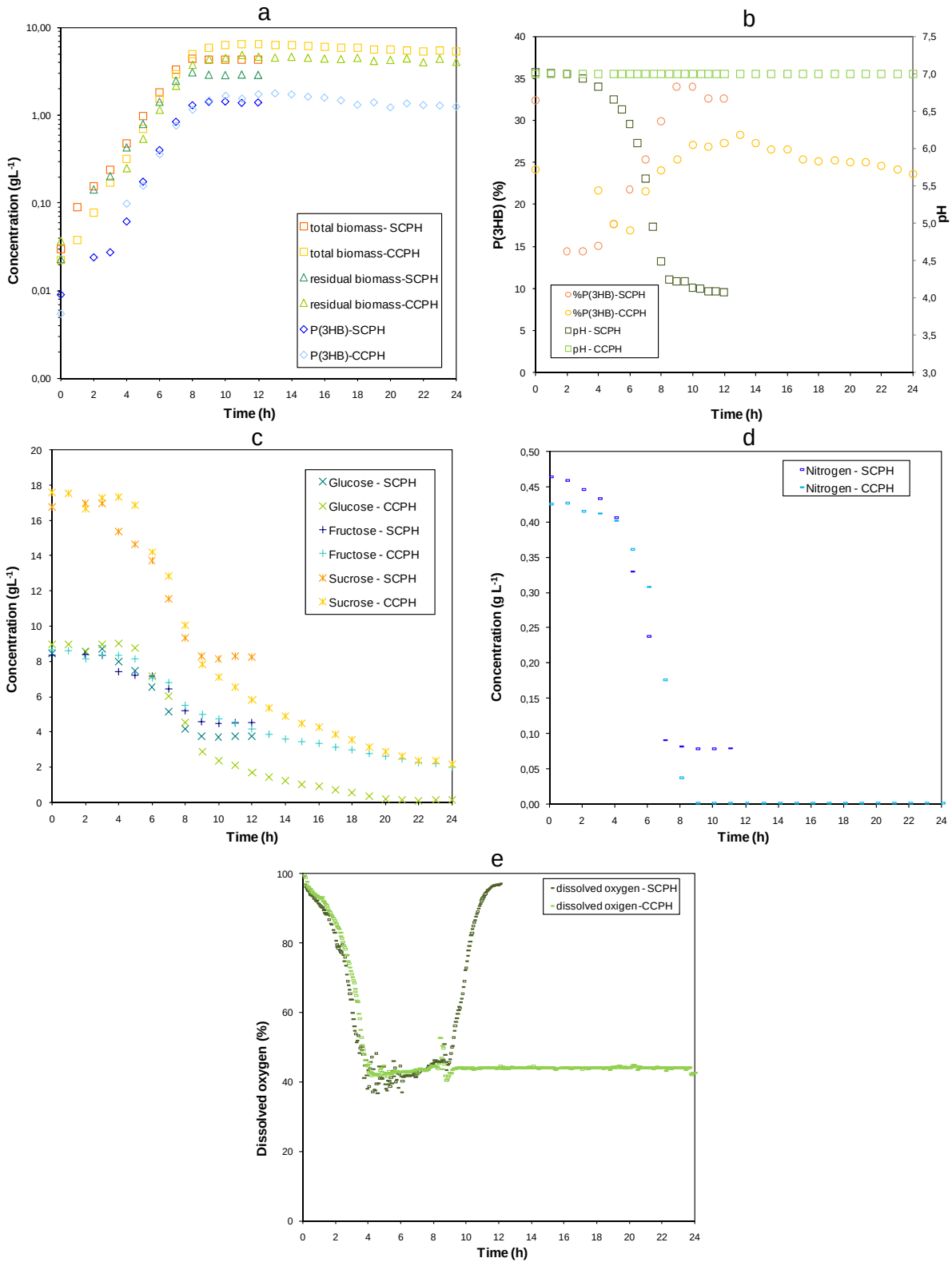


Figure 3: Time evolution of total biomass concentration, biopolymer accumulation and residual biomass (a), biopolymer accumulated and pH (b) sugar concentration (c), nitrogen concentration (d) and dissolved oxygen (e) throughout the culture without control pH (SCPH) and pH controlled (CCPH).

The values of parameters estimated using experimental data from the bioreactor with pH control are shown In Table 3. The simulation results showed good agreement with the experimental data, as pointed by the low value of the objective function.

Table 3: Parameter values for CCPH experiment.

Parameter	Unit	Values
α		2,0606
γ	1/h	0,0849
k_1		0,4053
k_2	1/h	-0,0079
k_d	1/h	0,0073
K_{SS}	g/L	0,1928
K_{SN}	g/L	0,0447
μ_m	1/h	0,7979
Y_{RN}		10,6314
Objective function		**0,1575**

5. Conclusions

Results of shaker experiments shown that *Bacillus megaterium* is able to produce large amounts of P(3HB) without nitrogen limitation. Results of shaker and bench-scale experiments realized the importance of a programmed pH control strategy, different from the used in CCPH experiment.

Therefore, more experiments need to be done in order to test new strategies for pH control in specific ranges (like as from 5 to 7), that can be able to generate similar results of P(3HB) accumulated in cells as the ones obtained in the shaker experiments.

6. Acknowledgements

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