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Evaluation of culture medium on poly(3-hydroxybutyrate) production by *Cupriavidus necator* ATCC 17697: application of the response surface methodology

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Abstract

Polyhydroxyalkanoates (PHA), of which polyhydroxybutyrate (PHB) is the most abundant, are polymers of bacterial origin used for various applications in the medical, industrial and agricultural fields. In the present study we worked on the selection, evaluation and improvement of the significant variables of the medium for the production of PHB by *Cupriavidus necator* ATCC 17697. In order to address the selection of the main factors and optimize the culture medium, a complete factorial experimental design based on the coupled response surface methodology, was presented. The model with the best adjustment of the variables turned out to be quadratic in fructose (C), linear in ammonium sulphate (N) and pH, with interaction in pH and phosphate solution (P), where the pH was the most significant ($p < 0.0001$) while the micro-elements solution could be neglected. Thus, optimum carbon concentration, adequate nitrogen limitation and interaction between initial pH and phosphate solution concentration are

important factors to ensure a high production of PHB. The optimal values of the selected variables were C = 20 g/l, N = 1.5 g/l, P = 8.75 g/l and pH 7.5. A maximum PHB production of 4.6 g/l, obtained under these conditions, increased almost 2.5 times. The polymer accumulated in the cytoplasm of *C. necator* ATCC 17697 in the form of granules showed an FTIR spectrum corresponding to that of commercial PHB.

Keywords: Mathematical biosciences, Microbiology

1. Introduction

Polyhydroxybutyrate (PHB) is a biopolymer belonging to the family of polyhydroxyalkanoates (PHAs). PHAs are biopolymers synthesized by a wide variety of microorganisms —as a carbon and energy reservoir under limiting conditions of essential nutrients— under an excess of the carbon source (Anderson and Dawes, 1990; Anjum et al., 2016). In terms of molecular weight, brittleness, stiffness, melting point, and glass transition temperature, the PHB homopolymer is similar to some of the more common petrochemical-derived thermoplastics, such as polypropylene (Barham et al., 1992; Koller, 2016). The upward marketing of PHAs is due to the excellent combination of their features: thermoplastic, biodegradable and, in some cases, biocompatible (Chen, 2009; Gao et al., 2011; Koller, 2018; Luef et al., 2015; Singh et al., 2019; Tan et al., 2016).

Culture media plays an important role in increasing the production of any metabolite along the microbial fermentation process. Thus, knowledge of the effect of the culture media variables (composition, pH, temperature, etc.) is crucial to decrease production costs. Conventionally, fermentations were improved or optimized by the variation of one component at a time. This approach is time-consuming and assumes that the effect of each process variable is independent on the effects of the others variables (Mu et al., 2009). In recent years, this approach has been replaced by statistical optimization methods that take into account the interaction of variables on to the process response. In most cases, this interaction is represented by a response surface coupled to the experimental design. The response surface methodology (RSM) is a type of statistical technique to design experiments by evaluating the relative importance of the independent variables, the potential interaction among them and determining the optimal conditions for desirable responses (Anderson-Cook et al., 2009). In the case of microbiological fermentation processes to get PHAs, RSM can be used to determine the composition of the culture medium that provides an optimal productivity of PHAs (Kennedy and Krouse, 1999; Yolmeh and Jafari, 2017).

Several research work have studied the effect of different media components using RSM to improve PHB production (Grothe et al., 1999; Nikel et al., 2005; Khanna

and Srivastava, 2005a; Tripathi et al., 2013; Aramvash et al., 2015). In all these cases, two experimental designs were considered: one to select the variables to be analysed and other to optimize these variables.

The main objective of this work is to analyze how the components of the culture medium affect the production of PHB by *Cupriavidus necator* ATCC 17697. RSM was used both to determine what components are significant in the production of PHB, and then optimize the concentration there of to maximize the production of PHB in 72 h. The experimental design includes all the variables of the culture medium, except the magnesium sulphate that is added in low concentration.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The strain used in all experiments is *Cupriavidus necator* ATCC 17697. It was stored in Viabank^{TM} cryovials at (-80) $\underline{o}$C. It was activated in a Petri dish containing nutrient agar and incubated at 30 °C for 48 hours. The inoculum, prepared with a loop of the reactivated strain in 100 ml of liquid TFL medium (Tryptone 5 g/l, yeast extract 5 g/l, fructose 1 g/l and potassium acid phosphate 1 g/l, pH 7), was grown for 24 hours at 30 °C and 150 rpm.

2.2. Medium and growing conditions

The culture of *C. necator* wild type usually employs fructose as the carbon source and ammonium sulfate as the nitrogen source (Wu et al., 2013) Therefore, in this work the PHB synthesis was carried out using a modified mineral salts medium (Barbosa et al., 2005) with: commercial fructose 15 g/l, ammonium sulphate 2 g/l, potassium dihydrogen phosphate 2 g/l, sodium monobasic phosphate 1.8 g/l, magnesium sulphate heptahydrate 0.5 g/l and microelement solution 2 ml/l. The microelement solution contained $FeSO_4$ 2 g/l, $MnCl_2 \cdot 4H_2O$ 0.03 g/l, $CaCl_2 \cdot 2H_2O$ 2 g/l, $CuCl_2 \cdot 2H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.1 g/l, H_3BO_3 0.3 g/l, $CoCl_2 \cdot 6H_2O$ 0.2 g/l, $NiCl_2 \cdot 6H_2O$ 0.02 g/l and $Na_2MoO_4 \cdot 2H_2O$ 0.03 g/l in 0.1 N HCl solution. The inoculum was sown at 5 % in 250 ml Erlenmeyer containing 60 ml of culture medium. Cultures were incubated at 30 °C for 72 hours and 150 rpm in a Vicking shaker pro.

2.3. Analytical methods

Cells were harvested by centrifugation with Presvac INS-DCA-300RTV centrifuge, washed twice with distilled water and air dried to constant weight at 80 °C in Numak DHG-9053A stove. The weight of the dried mass —named biomass and denoted X—was considered as the dry weight of the sample. The supernatant was used to determine the fructose and ammonium sulphate concentration. The concentration

of total organic carbon was determined using a Shimatzu Analyzer that operates in interface with TOC-Control L/V software. Ammonium sulfate was determined by the method of indophenol blue (Grasshoff et al., 2007).

Quantitative estimation of PHB was performed according to the modified method of Law and Slepecky (1960). PHB was converted into crotonic acid when heated in H_2SO_4 80%. The absorbance of crotonic acid was measured at 234 nm with Perki- nElmer UV/Vis spectrometer Lambda 35. The residual biomass (X_R) was defined as

$$X_R = X - PHB \tag{1}$$

where PHB denotes the PHB weight.

The biomass and PHB production yields were calculated in g/g according to Eqs. (2) and (3), respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta C} \tag{2}$$

$$Y_{P/S} = \frac{\Delta PHB}{\Delta C} \tag{3}$$

where ΔC is the concentration of fructose consumed in g/l; ΔX and ΔPHB —in g/l — are the differences between the biomass and PHB production, respectively, at the end and the beginning of the fermentation.

PHB yield as a function of biomass ($Y_{P/X}$) and polymer productivity (P_{PHB}) were calculated according to the Eqs. (4) and (5), respectively.

$$Y_{P/X} = \frac{\Delta PHB}{\Delta X} \tag{4}$$

$$P_{PHB} = \frac{\Delta PHB}{\Delta t} \tag{5}$$

where Δt is the fermentation time for the ΔPHB production.

2.4. Experimental design

RSM was applied considering the PHB production by *C. necator* after 72 hours of fermentation as the design response. The variables were: fructose concentration, as the sole source of carbon (C), ammonium sulphate concentration, as the sole source of nitrogen (N), the initial pH of the culture medium, the concentration of the phos- phate solution (P) (as the sum of the concentrations of dihydrogen potassium phos- phate and monobasic sodium phosphate) and the concentration of the micro- elements solution (M). A full factorial design (FFD) of five variables in two levels (+ and -) with a central point was used (Table 1).

Table 1. Full Factorial Design variables: fructose concentration (C); ammonium sulphate concentration (N); initial pH of the medium; concentration of the phosphate solution (P); concentration of the microelement solution (M).

Variable	Unit	Levels		
		-	0	+
C	g/l	15	20	25
N	g/l	1.5	2.25	3
pH	-	6.5	7	7.5
P	g/l	4	8	12
M	ml/l	1	2	3

Each condition was replicated in duplicate trials for each case, except for the central point for which six replicates were considered to adequately assess the variance of the results.

2.5. RSM

The experimental values of the PHB concentration for the culture media summarized in Table 1 were analysed by the response surface regression procedure (Yolmeh and Jafari, 2017) using the following second-order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \tag{6}$$

where Y_i is the predicted response by the model, x_i the independent variables analysed, β_s the estimated coefficients: β_0, the offset term, β_i, the linear coefficients, β_{ii}, the quadratic coefficients and β_{ij}, the interaction coefficients. Analysis of variance (ANOVA) was used to estimate these statistical parameters. The significance of Eq. (6) and its terms were evaluated by F-test and the fitting quality by the correlation coefficient, R^2. The Minitab 14 software was used for calculations and graphs of the surface plots.

2.6. Analysis and validation

3D graphics were used to analyse the optimized components of the medium that influences the design response. The validation of the optimum conditions predicted through RSM is crucial (Yolmeh and Jafari, 2017). For this purpose, it is important that the evaluation of the optimized culture medium is performed under the same experimental conditions as those used to get the experimental data for the RSM. Therefore, for the validation 6 fermentations in Erlenmeyer were analyzed: one with the culture medium of the maximum PHB production and two with PHB production around the maximum, each condition was performed in duplicate. It is worth

to notice that fermentations in bioreactors cannot be considered for the validation since they lead to PHB productions different than our experimental data because the control strategies are modified, e.g. pH regulation, agitation, aeration, etc (Kennedy and Krouse, 1999).

2.7. Transmission electron microscopy (TEM)

Cells were mixed with 2.5% glutaraldehyde in 0,1M phosphate buffer (pH 7.4), kept at 4 °C for 4hs. And then washed twice with the buffer. Cells pellets were stained with 2 ml 1% osmium tetroxide in phosphate buffer during 1 h at 4 °C, washed twice in deionized water and dehydrated with a graded ethanol series, followed by 100% acetone. The treated cells were embedded in "Durcupan" resin; the resin was polymerized at 60 °C for 72 h. Embedded cells were cut into ultra-fine sections 70-90 nm with a glass knife and were later collected onto copper grids; these grids containing ultra-fine sections of the cells were immersed in uranyl acetate and lead citrate solutions, respectively, for 15 min. Sections were observed using a Zeiss EM109T transmission electron microscope in LANAIS-MIE, Faculty of Medicine, University of Buenos Aires.

2.8. Fourier transform infrared spectroscopy (FTIR)

PHB was extracted from the dried cells with chloroform at 70 °C during 24 hours using a Soxhlet extractor. The extract was then concentrated using a rotary evaporation Senco R206B and the polymer was precipitated in 10 volumes of ice-cold methanol at 4 °C. PHB polymer was purified by precipitation with methanol at 4 °C and filtered through a sintered ceramic filter. The polymer was left to dry. Fourier transform infrared spectroscopy (FTIR) in the attenuated total reflectance mode (ATR) on ZnSe crystal was used for the qualitative analysis of PHB polymer. Infrared spectrum of the isolated polymer and the commercially available Biocycle[®] 1000 - PHB polymer (PHB Industrial S/A, Brasil) were obtained at 400-4000 cm^{-1} on a FT-IR Nicolet 8700 spectrophotometer. 32 scans, resolution of 4 cm^{-1} and interval of 2 cm^{-1} were used.

3. Results and discussion

### 3.1. Typical profile of *C. necator* fermentation

Fig. 1 shows the profile of the *C. necator* fermentation; the consumption of fructose, ammonium sulphate, pH decrease, biomass production and PHB accumulation were monitored at regular time intervals.

According to the residual biomass curve (calculated by Eq. (1)), the exponential phase begins after a lag phase of 6 hours while the stationary phase appears between
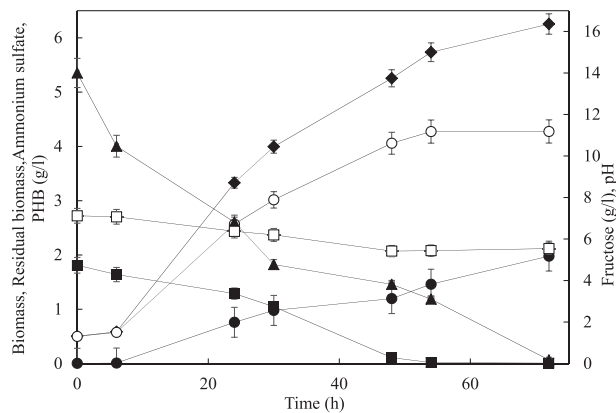
Fig. 1. Typical profile of *C. necator* ATCC 17697 in culture medium with fructose 15 g/l, incubated at 30 °C, 150 rpm, for 72 hours for: () total biomass, (○) residual biomass and (●) PHB, and changes in (■) ammonium sulfate, (▲) fructose and (□) pH.

50 and 72 hours after the beginning of the fermentation. The consumption of fructose and ammonium sulphate accompanies the bacterial growth and the consequent accumulation of the polymer. The pH decreases throughout the fermentation process. PHB accumulation begins with the exponential phase and continues to increase in the stationary phase, when the residual biomass remains constant. In the exponential phase, the ammonium sulphate is already exhausted and a significant consumption of fructose is observed. The total biomass curve shows an increase, from 53 to 72 hours, due to the accumulation of PHB inside the cells, when the residual biomass remains constant. At this phase, cell division has already ended and the cells are in a metabolism of intracellular polymer accumulation, induced by limitation of N and excess of the carbon source (López-Cuellar et al., 2011). Therefore, the 72-hour incubation time was selected for the experimental design of this work; this time gave the maximum PHB production and full consumption of the substrate. The TEM
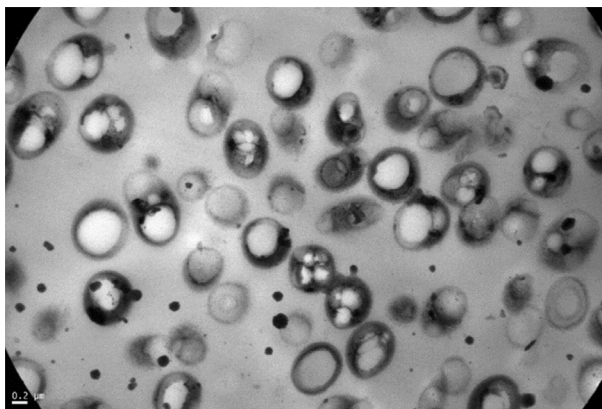
Fig. 2. TEM of *C. necator* ATCC 17697 cells cultivated during 72 h (20000 X).

photograph of *C. necator* ATCC 17697 grown on fructose as a carbon source showed the internal granular structure of PHB polymer produced (Fig. 2).

3.2. FFD experimental design

The FFD of five variables analysed at two levels generated a design matrix of 32 experiments plus the central point. Statistical analysis of the PHB values measured in duplicate was performed. Table 2 shows the values of the coefficients in coded and uncoded units of the second order polynomial equation. ANOVA analysis was used to estimate the statistical parameters, with significance value $p < 0.05$.

The goodness of the model, can be checked by the percentage variability in the y values of experimental data and values calculated by the model (R^2) and by adjusted R^2 (Adj. R^2). Adj. R^2 is the value of R^2 adjusted down for a higher number of

Table 2. Statistical analysis for the variables of the experimental design: fructose concentration (C); ammonium sulphate concentration (N); initial pH; concentration of the phosphate solution (P); concentration of the microelements solution (M). The coefficients of the variables are in coded and uncoded units.

Variable	Coefficients in coded units	Standard error	p value	Coefficients in uncoded units
Constant	4.1493	0.19137	<0.001	-39.3042
C	0.0726	0.05652	0.204	2.5411
N	-0.5674	0.0586	<0.001	-2.8322
pH	0.5052	0.0586	<0.0001	2.0879
P	0.0898	0.0586	0.131	1.4618
M	0.0905	0.0586	0.128	-1.3090
C*C	-1.7424	0.28408	<0.001	-0.0697
N*N	-0.1797	0.21797	0.413	-0.3195
C*N	0.0027	0.0586	0.963	0.0007
C*pH	0.1007	0.0586	0.091	0.0403
C*P	-0.0490	0.0586	0.407	-0.0025
C*M	-0.0064	0.0586	0.913	-0.0013
N*pH	-0.1090	0.0586	0.068	-0.2907
N*P	-0.1143	0.0586	0.056	-0.0380
N*m	0.0651	0.0586	0.271	0.0868
pH*P	-0.3839	0.0586	<0.001	-0.1920
pH*M	0.0767	0.0586	0.196	0.1534
P*M	0.0780	0.0586	0.188	0.0195

$R^2 = 85.10\%$ Adj. $R^2 = 80.60\%$ SD = 0.4688

Adj. $R^2 = 80.6\%$ means that the model fitted very well and can predict satisfactorily the experimental response.

variables in the model. Normally, the model has a very high correlation if $R^2 > 0.9$ (90%) and a high correlation if $0.7 < R^2 < 0.9$ (Haaland, 1991; Kennedy and Krouse, 1999).

It is important to note that not all variables affect the performance in the same manner. A strong, a medium or no influence at all of a variable on the output performance is given by the p value: the lowest value, the highest significance (Daneshi et al., 2010). The model with the best fit to the experimental data was quadratic in C, linear in N and pH and with interaction in pH and P, being the pH the most significant ($p < 0.0001$). The interaction between N and P is in a limit situation ($p = 0.056$). The variable M was not significant, along with all its interactions. Therefore, a minimum and necessary concentration of microelement solution in the culture medium of *C. necator* is sufficient to obtain an optimal concentration of biomass and PHB. This result differs from that obtained with a similar design for *Alcaligenes latus*, where the solution of microelements was one of the most significant variables (Grothe et al., 1999).

Coded variables, obtained by standardization of the coefficients, allow to determine how much they influence the response. In the Eq. (6) the constant term indicates the production of PHB at the centre of the design. The variable with the largest standardized coefficient was the quadratic term of carbon (-1.7424). N and pH have similar standardized coefficients, although of opposite signs (-0.5674 and 0.5052, respectively); this means that an increase in the pH or adecrease in the N concentration have the same influence on the polymer production within the limits of the design.

3.3. Optimization by response surface methodology

The fitted polynomial equation was represented as three-dimensional surface plots to visualize the relationship between the response and the experimental levels of each factor used in the design. Regarding the second order polynomial equation, PHB production response surfaces were built using two significant variables, with fixed values for the others.

Results summarized in Table 2 are illustrated in Fig. 3. The production of PHB is enhanced by increasing pH values (Fig. 3a) and decreasing N values (Fig. 3b).

The only significant interaction is between P and pH. In fact, when the pH value is minimal and the concentration of P increases, the production of PHB also increases. This positive slope decreases as the pH increases. When the pH reaches its highest value, the slope of PHB production as a function of P becomes negative (Fig. 2c). The factor associated to the quadratic contribution of the carbon source has its maximum in the vicinity of the central point. Thus the maximum PHB production occurs when the fructose variable has a value of 20 g/l (Fig. 3 a, b).
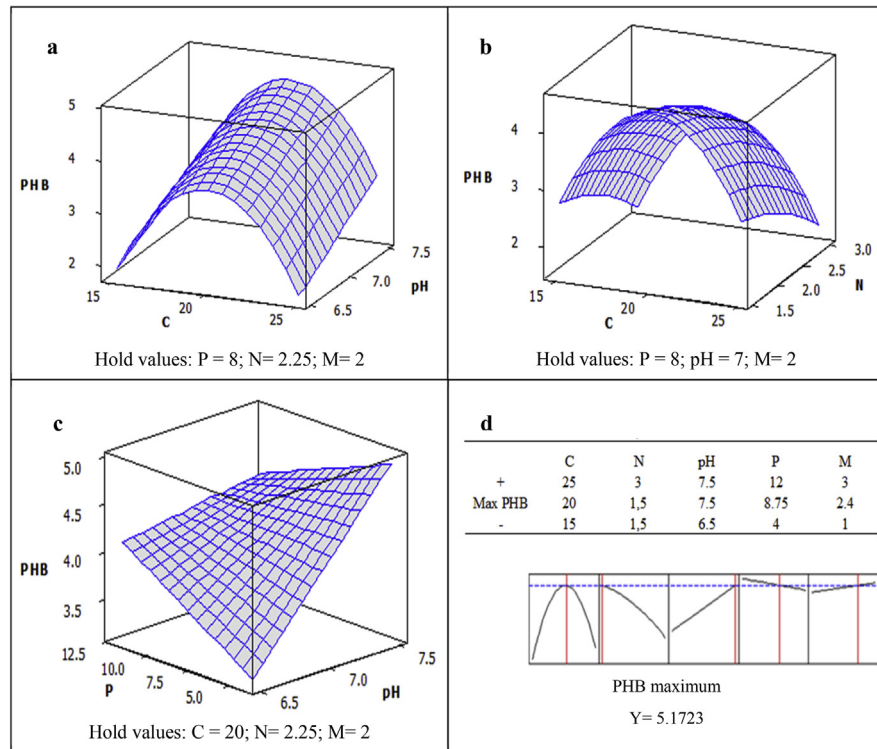
Fig. 3. PHB production as a function of C and pH (a); C and N (b) P and pH (c) and coordinates for the maximum production area of PHB (d). For a certain C concentration: PHB production increases as pH increases and decreases as N content increases; no interaction between C and pH or N was found. At low pH the PHB production is favored by the increase of P; however, at the highest level of pH, this relationship changes, becoming inverted.

The fitting model has a maximum at C = 20.412 g/l, N = 1.50 g/l, pH = 7.5, P = 8.74 g/l, M = 2.45 g/l with a predicted maximum value of PHB = 5.17 g/l ± 0.62 g/l. It is noticed that the point of optimum PHB production is within the experimental region (Fig. 2d).

3.4. Model validation

To validate the model, three points were considered: the maximum and two points in its environment. The results are within the limits of the confidence interval for the predicted value (Table 3).

Table 4a shows the matrix of the full factorial design (16 points), plus the optimized and validated point. The variable M (microelements) was eliminated from the matrix, as it was not significant, according to the results shown above. Table 4b shows the values of the measurements of pH and fructose change, biomass and PHB production at 72 hours of fermentation of the experimental points. Table 4c shows the calculated yields $Y_{X/S}$ (Eq. (2)), $Y_{P/S}$ (Eq. (3)), $Y_{P/X}$ (Eq. (4)) and productivity (Eq. (5)) of each of the processes.

Table 3. Model validation. PHB production predicted by the RSM compared to the experimental values for the optimum composition according to the FFD and other two surrounding compositions. Fructose concentration (C); ammonium sulphate concentration (N); initial pH of the medium; concentration of the phosphate solution (P); concentration of the microelement solution (M).

C	N	pH	P	M	Predicted value PHB (g/l)	Experimental Value PHB (g/l)
20	1.5	7	8	2	4.35 ± 0.59	4.39 ± 0.07
20	3	7	8	2	3.40 ± 0.59	3.50 ± 0.29
20	1.5	7.5	8.75	2	5.17 ± 0.62	4.59 ± 0.04

The experimental value of the optimized point —4.6 g/l PHB— corresponds to a PHB content of 70 %; it is the maximum value obtained experimentally throughout the design (Table 4b).

3.5. Analysis of yields and productivities at the experimental values

Hydrogen ions (H^+) are produced during biomass growth, decreasing the pH of the mineral medium solution; thus, the medium needs to be neutralized using alkali or phosphate buffers to maintain the pH at the optimum level for the growth of *C. necator* (Mozumder et al., 2014). By RSM it was determined that the interaction between the initial pH and phosphate buffers is significant. The influence of these variables on the final pH, fructose consumption, biomass and PHB production and the yields and productivity of the experimental values are analyzed below.

In all odd experiments the concentration of P is at its lowest level. For experiments 1, 5, 9 and 13 —where the initial pH is 6.5— the final pH reaches very low values, close to 4, with the minimum biomass production and, as a consequence, a low production of PHB: 2.2—2.5 g/l and 1.1—1.5 g/l, respectively. This also coincides with a low consumption of fructose — between 7.8 and 10 g/l. Therefore, an initial pH of 6.5 and the rapid decrease to a pH close to 4 limits bacterial growth and PHB production. This is according to a study of Beaulieu et al. (1995), who reported that the growth of *C. necator* was inhibited when the pH descended to less than 5.4.

However, in experiments 3, 7, 11 and 15 where the initial pH is 7.5, a higher biomass and PHB production —4.8-6.6 and 2.1—4.5 g/l, respectively— and a high fructose consumption —14.3—18.1 g/l— was measured. Thus starting at higher pH, it remains virtually constant along the process and exhibit a sudden drop towards the end of the fermentation, without affecting the production of biomass.

In even-numbered experiments, where the P level is high, there is not a large decrease in pH independent of the initial pH. Therefore, the phosphate solution is

Table 4. a. Design matrix: Exp, number of experiment (0, center point, Op; optimized point), Fructose concentration (C); ammonium sulphate concentration (N); initial pH of the medium; concentration of the phosphate solution (P); concentration of the microelements solution (M). b. Analytical measurements after 72 hours: change in pH of the medium (Δ pH), fructose consumed (ΔC), total biomass (X), PHB production (PHB). c. Yields of fermentation processes ($Y_{P/X}$, from biomass to product; $Y_{P/S}$, from substrate to product, $Y_{X/S}$, from substrate to biomass) and polymer productivity (P_{PHB}).

a					b				c			
Exp	C	N	pH	P	ΔpH	ΔC	X	PHB	$Y_{P/X}$	$Y_{P/S}$	$Y_{X/S}$	P_{PHB}
	g/l	g/l		g/l		g/l	g/l	g/l	g/g	g/g	g/g	g/l/h
0	20	2.25	7	8	0.9	18.7	6.8	4.0	0.59	0.21	0.36	0.06
1	15	1.5	6.5	4	2.3	8.5	2.4	1.3	0.55	0.16	0.28	0.02
2	15	1.5	6.5	12	0.4	14.7	4.2	3.1	0.73	0.21	0.29	0.04
3	15	1.5	7.5	4	1.2	14.3	5.2	3.1	0.60	0.22	0.36	0.04
4	15	1.5	7.5	12	0.5	13.3	5.1	2.8	0.54	0.21	0.38	0.04
5	15	3	6.5	4	2.2	7.8	2.3	1.3	0.58	0.17	0.29	0.02
6	15	3	6.5	12	1.3	13.8	4.7	1.4	0.30	0.10	0.34	0.02
7	15	3	7.5	4	2.9	14.5	5.2	2.4	0.46	0.17	0.36	0.03
8	15	3	7.5	12	1.2	13.8	4.7	1.0	0.21	0.07	0.34	0.01
9	25	1.5	6.5	4	2.2	8.9	2.5	1.4	0.57	0.16	0.28	0.02
10	25	1.5	6.5	12	0.4	10.6	4.8	2.8	0.58	0.27	0.46	0.04
11	25	1.5	7.5	4	1.3	18.1	6.6	4.5	0.68	0.25	0.36	0.06
12	25	1.5	7.5	12	0.5	15.5	5.4	2.8	0.51	0.18	0.35	0.04
13	25	3	6.5	4	2.2	10.0	2.2	1.1	0.51	0.11	0.22	0.02
14	25	3	6.5	12	0.6	11.7	4.0	1.1	0.27	0.09	0.34	0.01
15	25	3	7.5	4	3.3	15.2	4.8	2.1	0.44	0.14	0.31	0.03
16	25	3	7.5	12	0.9	18.5	8.0	2.4	0.30	0.13	0.43	0.03
Op	20	1.5	7.5	8.75	0.9	14.7	6.5	4.6	0.70	0.31	0.44	0.06

acting as a pH regulator. In all these experiments, there is a good biomass production between 4.5 and 8 g/l, but with different $Y_{P/X}$ yields. In assays 6, 8, 14 and 16, where N and P are at the highest level, the $Y_{P/X}$ yield is at least 0.21−0.30 g/g; this happens because there is no limiting nutrient in the culture medium that induces PHB production.

The optimum initial pH was 7.5; combined with the P solution in a concentration of 8−12 g/l it allowed to have a pH close to 7 during the whole fermentation. Also, the P-pH interaction was significant. However, the concentration of P at high levels (12 g/l) lowers PHB production. Therefore, it is important that the concentration of P is

at an adequate level, since it regulates the pH and at the same time is not too high to inhibit the production of the biopolymer. This ensures both microbial growth and the accumulation of PHB.

Nitrogen limitation promotes PHB production in *C. necator*, however, it should be sufficient to allow a proper microbial growth (Khanna and Srivastava, 2008, 2005b; López-Cuellar et al., 2011). In this work it was determined that 1.5 g/l of ammonium sulphate lead to get a good biomass production with the maximum yield of PHB.

The quadratic model term C turned out to be the variable with the largest coefficient. The optimum carbon concentration for the production of PHB in a batch system for *C. necator* was 20 g/l of fructose. This is consistent with the maximum fructose consumption measured (18.7 g/l), even for the experiments with the highest initial fructose concentration (25 g/l). These results correspond to previously published values (Kim et al., 1994; Mozumder et al., 2014). Mozumder et al. showed that the specific growth rate of *C. necator* significantly lowers for carbon source concentrations greater than 20 g/l.

PHB content is between 1 and 3 g/l in most designs, except for the points: central, 11 and optimized, where it is equal to or greater than 4 g/l. The maximum production of PHB correspond to assay 11 the optimum point; 4.5 and 4.6 g/l, respectively. In these assays, the maximum $Y_{P/X}$ yields were 0.68 and 0.70 g/l due to the optimum concentrations of C and N.

At the optimum point all yields and productivity calculated are maximum; $Y_{P/S}$ shows the greatest difference with respect to the other experimental values. This fact indicates a better use of the carbon source to produce the biopolymer with less substrate waste.

In the initial experiments the PHB production was 1.9 g/l. It increased to 4.6 g/l, the PHB production raised by almost 2.5 times after optimizing the composition of the culture medium by RSM.

3.6. FTIR analysis

FTIR spectroscopy is an ordinary chemical technique used for the qualitative study of molecular structures; the FTIR spectrum of a sample represents its overall chemical composition. By means of this technique, the presence of the main functional groups of the PHB polymer could be verified. Fig. 4 shows the FTIR spectrum of the PHB samples produced from the optimized medium by *C. necator* ATCC 17697 and the commercially available PHB.

The remarkable peak at 1720 cm^{-1}, points out the presence of ester carbonyl (C=O) groups, which are a characteristic of PHB (Aramvash et al., 2015). Other bands located near 1276 and 1160 cm^{-1} were attributed to C-O-C groups. The C-O bonds
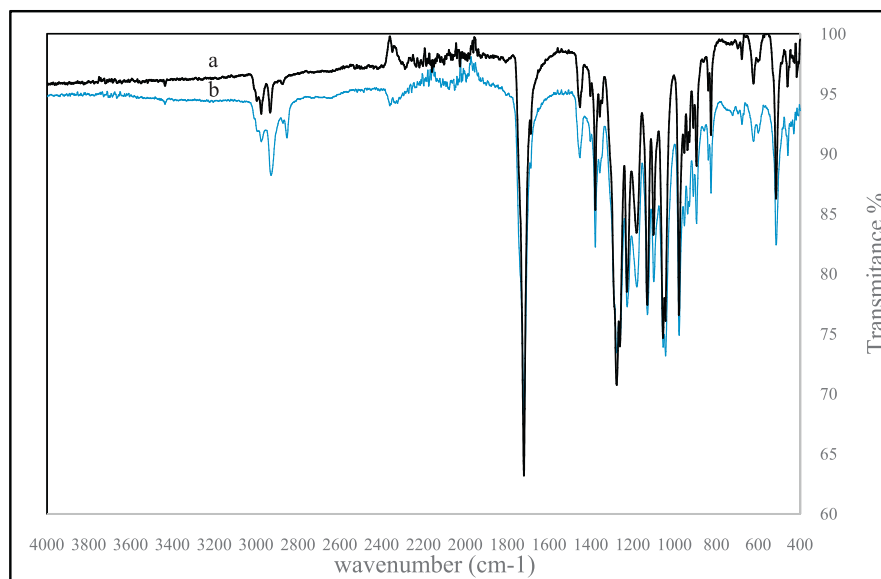
Fig. 4. FTIR spectra of commercially available PHB (a) and PHB produced by *C. necator* ATCC 17697 (b).

were evidenced by the bands located at 1050 and 979 cm^{-1}. The C-H stretch bonds in the polyester were assigned to the bands located in the spectral region around 2900 cm^{-1}. The obtained FTIR absorption peaks from the culture were in agreement with the corresponding spectra to pure PHB. Based on the above results, it was concluded that the extracted compound from *C. necator* ATCC 17697 should be PHB.

4. Conclusion

It was possible to determine the optimal operating conditions for PHB production using *Cupriavidus necator* ATCC 17697. Afull factorial design coupled to the RSM was used as a method to determine the main factors and, at the same time, to optimize the culture medium.

In this study, the model with the best fit was quadratic in C linear in N and pH, with interaction in pH and P, where the pH was the most significant ($p < 0.0001$), and M was not significant. Optimum carbon concentration, adequate nitrogen limitation and interaction among the initial pH and the concentration of the phosphate solution were important factors to ensure a high PHB production. The optimal values of the selected variables were C = 20 g/l, N = 1.5 g/l, P = 8.75 g/l and pH = 7.5. Using the optimized medium, the PHB concentration reached 4.6 g/l, that is, increased almost 2.5 time. Thus RSM became a suitable method to optimize the media. The polymer accumulated in the cytoplasm of *C. necator* ATCC 17697 in the form of granules showed an FTIR spectrum corresponding to that of commercial PHB.

Declarations

Author contribution statement

Daiana Nygaard: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Oxana Yashchuka: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Élida B Hermida: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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