Application of Experimental Design and Canonical Analysis of Response Surfaces to the Optimization of Poly(3-hydroxyalkanoates) Production by *Pseudomonas aeruginosa* 42A2

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The aim of this work was to optimize the culture medium for biomass and PHA production by *Pseudomonas aeruginosa* 42A2, using a five-level-three-factor central composite rotatable design (CCRD) combined with response surface methodology (RSM). A total of 23 sets of experiments was developed to obtain the second-order polynomial equations that were used to predict biomass and PHA production in terms of three independent variables: carbon source, nitrate and phosphate concentrations.

Canonical analysis of the response surface models showed that the optimum medium composition differed for the production of biomass and PHA. When the models were validated experimentally by culturing *Pseudomonas aeruginosa* 42A2 in the optimal media, similar values to the predicted ones were obtained for the biomass (18.73 g L^{-1}) and PHA (4.52 g L^{-1}) concentrations.

Moreover, central composite rotatable design and canonical analysis of the response surfaces proved to be useful tools for determining the optimum composition of the culture medium.

Key words:
Bioprocessing, fermentation, optimization, response surface methodology, canonical analysis, polyhydroxyalkanoates

Introduction

Many attempts to create bio-plastics from renewable resources have been made in recent years. The main aim of these studies has been to develop products competitive with synthetic polymers. Two main strategies have been studied to produce reasonable yields of these polymers: transgenic plant cells[1] and the well known bacterial fermentation process.[2]

Among the various candidates for biodegradable polymers, polyhydroxyalkanoates (PHAs) have attracted considerable attention because of their similar properties to those of some common conventional plastics.[3–5] PHAs are structurally accumulated as discrete intracellular cytoplasmic granules in bacterial cells as a result of the metabolic stress created by an unbalanced growth when an essential nutrient such as nitrogen, phosphorus, magnesium or oxygen is available only in limiting concentrations in the presence of excess carbon source.[3,6,7]

The main limitations in the production of PHAs are the special growth conditions required for the synthesis of the compounds, the difficulties involved in synthesizing them from inexpensive precursors and the high cost of their recovery.[8] To reduce the production cost of medium-chain-length PHAs (mcl-PHAs), the use of media containing agro-industrial wastes such a palm-oil, molasses, whey, hemicellulose, corn, cassava, glycerol-rich biodiesel coproduct systems, meat-and-bone meal and ice cream residue, among others, have been studied.[4,9–16] In this context, waste frying oils, sub-products from the vegetable oil refining process and linseed oil were used for the production of mcl-PHAs by *Pseudomonas aeruginosa* 42A2.[11,17,18]

However, the polymer content obtained with agro-industrial wastes is considerably lower than that obtained using purified carbon substrate. Therefore, there is a need to develop more efficient and appropriate bioprocess strategies for producing these polymers from cheap carbon sources.[19–21] Thus, optimization of the fermentation medium is necessary and it has often been used to enhance the

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yield and productivities of many biotechnological processes.[22,23]

Response surface methodology (RSM) is a collection of statistical techniques for experiment design, model development, factor evaluation and for identifying optimum conditions. The underlying principles of RSM are simple, and the method is powerful and highly efficient for the experimental attainment of optimum operation conditions. RSM could overcome the shortcomings of the classical or empirical methods, such as the "one-factor-at-a-time-technique", which is a time-consuming process and incapable of identifying the optimal overall conditions.[22]

Analysis of the eigenstructure of response surfaces is known as the *canonical analysis* of the response surface model, it was introduced by George E. P. Box and co-workers in their pioneering works more than 50 years ago.[24] We agree with Carlson & Carlson that, scanning the recent literature on process development, revealed that this technique is either unknown to researchers working in industrial microbiology or purposely overlooked.[25] To our knowledge, only 41 references were found in the last 10 years.

A canonical analysis facilitates interpretation of the response surfaces by examining the overall shape of the curve and establishing whether the estimated stationary point is a maximum, a minimum or a saddle point. Canonical analysis can be used to ascertain: i) the shape of the surface (a hill, a valley, a saddle surface or a flat surface), ii) whether there is a unique optimum combination of factor values and iii) to identify which factor or factors represent the most sensitive predicted response.[26]

The aim of this paper was to optimize the culture medium for producing biomass and PHA by *Pseudomonas aeruginosa* 42A2 using RSM, a central composite rotatable design (CCRD) and canonical analysis. An industrial oil subproduct, named WEICHOL 3/96 V, which is a mixture of oils and vegetable fats of the triglycerides family, composed of palm olein and rapeseed oil, was used as a carbon source. Two responses were measured: biomass (Y_1) and PHA (Y_2) concentrations and the considered factors were carbon source (WEICHOL), nitrate ($NaNO_3$) and phosphate (K_2HPO_4/KH_2PO_4 ratio 2 : 1).

Materials and methods

Microorganism

The aerobic gram-negative rod-shaped bacterium isolated from oily-residues contaminated waters and identified as *Pseudomonas aeruginosa* 42A2 (NCIMB 40045) was used in the present study.[11]

The strain was maintained on TSA (Trypticase Soy Agar; Difco) slants at 30 °C and was kept after 24 h incubation at 4 °C and subcultured every month. Alternatively, the strain was preserved in cryovials (EAS Laboratoire, France) at –80 °C.

Carbon substrate and chemicals

The carbon source used in this study was WEICHOL 3/96 V, and was a gift from Industrial Química Lasem, S.A. (Castellgalí, Barcelona). Fatty acid composition (w/w): 0.85 % myristic acid (C14 : 0); 27.2 % palmitic acid (C16 : 0); 2.7 % palmitoleic acid (C16 : 1Δ9); 4.54 % estearic acid (C18 : 0); 14.3 % linoleic acid (C18 : 2Δ9,12), and 50.4 % oleic acid (C18 : 1Δ9).

Chemicals were all of analytical grade. SDS (Peypin, France) supplied the organic solvents. Panreac (Barcelona, Spain) supplied other chemical products, all of ACS quality. ADSA (ADSA, Barcelona, Spain) and Pronadisa (Barcelona, Spain) supplied the microbiological media.

Cultivation medium

The cultivation medium used for the production of biomass and PHA was a minimal basal medium containing (in g L^{-1}): $CaCl_2$ (0.04), KCl (0.4), $MgSO_4 \cdot 7H_2O$ (2), $FeSO_4 \cdot 7H_2O$ (0.012), and with 0.2 mL/L of the following trace elements solution (mg/100 mL): H_3BO_3 (148); $CuSO_4 \cdot 5H_2O$ (196); $MnSO_4 \cdot H_2O$ (154); Zn $SO_4 \cdot 7H_2O$ (307) and Na-MoO_4 $2H_2O$ (15). Since the carbon source, nitrogen and phosphorus are essential nutrients of the medium and influence the growth and accumulation of PHA, these three constituents, WEICHOL 3/96 V, $NaNO_3$ and K_2HPO_4/KH_2PO_4 (2 : 1 in weight), were taken for optimization of their concentration using the RSM. The components were added following the experimental design as mentioned in Tables 1 and 2. WEICHOL 3/96 V and the salt solution were sterilized separately at 121 °C and 1 bar for 20 min and then cooled and aseptically reconstituted at room temperature prior to use. The final pH was adjusted to 6.8 with 0.1M NaOH/0.1N HCl.

Inoculum preparation, shake flask experiments and cultivation conditions

The inoculum preparation culture and the final optimization experiments were carried out in baffled Erlenmeyer flasks (500 mL capacity) with 50 mL minimal basal medium containing different concentrations of the three constituents (WEICHOL 3/96 V, $NaNO_3$ and K_2HPO_4/KH_2PO_4). Throughout the study, all flasks were inoculated with a 2 % (v/v) cell suspension on sterile NaCl 0.9 % of an overnight culture grown at 30 °C on TSA (Trypticase Soy Agar, Difco) and adjusted by turbidimetry

($A_{540} = 2.0 \pm 0.2$) against sterile NaCl 0.9 % in a UVIKON 922 spectrophotometer (Kontron Instruments, Milan, Italy). Incubations were carried out on an orbital shaker for 48 h at 150 rpm rotational speed and 30 °C. All the experiments were carried out in triplicate.

Analytical methods

Samples were taken at given intervals and analyzed to measure the concentrations of biomass and PHA.

Determination of biomass

The biomass content on dry cell weight (DCW) was evaluated gravimetrically (g L^{-1}) as follows: 15 mL culture samples were aseptically removed from the flasks at 48 h and centrifuged at 18000 rpm for 20 min at 4 °C (Kontron, Milan, Italy). The supernatant was discarded, and the cell pellet was washed with deionized water/hexane (10 : 1) to remove contaminating residual carbon source and residual culture medium, then washed twice with deionized water and recovered (18000 rpm, 5 min, 4 °C). The washed cells were dried at 100 °C for 24 h in a hot air oven to a constant weight in pre-weighed tubes and then cooled in a desiccator and weighed. All measurements were made in triplicate. Cell concentration was expressed as g L^{-1}.

Determination of PHA

The PHA concentration of bacterial cells was determined by gravimetric quantification after a recovery treatment as follows. PHA was extracted and purified using 15 mL of culture samples. Cells were harvested aseptically after 48 h by centrifugation at 18000 rpm for 20 min at 4 °C (Kontron, Milan, Italy) and washed with deionized water/hexane (10 : 1) to remove contaminating residual carbon source and residual culture medium, then washed twice with deionized water and recovered (18000 rpm, 5 min, 4 °C) and lyophilized in Cryodos-50 (Telstar, Terrassa, Spain) at –56 °C and 10^{-2} mbar. The lyophilized biomass was extracted with hot chloroform in a Pyrex tube and heated for 3 h at 100 °C. The chloroform solution was filtered to remove any cell debris and concentrated by rotary evaporation

(Bücchi, Flawil, Switzerland). PHA was purified by precipitation of the chloroform solution through drop-wise addition to a cold methanol.[11,17] The methanol-chloroform mixture was decanted and the pure polymer was washed with fresh iced methanol. The polymer was re-dissolved in chloroform, concentrated to dryness under vacuum (Bücchi, Flawil, Switzerland), and weighed. The purified polymer was kept under N_2 at –20°C. All tubes were pre-weighed and all measurements were made in triplicate. PHA concentration was expressed as g PHA/L.

Experimental design

A five-level-three-factor central composite rotatable design (CCRD), requiring 23 experiments, was employed in this study.[24] The fractional factorial design for three factors consisted of $2^3 = 8$ factorial points at +1 and –1 level, 2*3 = 6 axial points (2 axial points on the axis of each design variable at a distance of +1.68 and –1.68 from the center, and 9 central points for the estimation of a pure error. All experiments were carried out in triplicate and the data shown here are the mean values. To avoid bias, the experiments were performed in a random order (overall randomization).

The dependent variable (responses) chosen in the present work were: biomass production expressed as concentration of biomass in g L^{-1} (Y_1) and PHA production expressed as concentration of PHA in g L^{-1} (Y_2).

The selected independent variables (factors) were: X_1 : carbon source, X_2 : nitrogen and X_3 : phosphorus. The different range and levels of the selected factors applied in this study are shown in Table 1 in terms of coded and natural units. The variables were coded according to the following equation:

$$x_i = \frac{\left(X_i - X_i^*\right)}{\Delta X_i} \qquad (1)$$

where x_i is the coded value of the ith independent variable, X_i is the corresponding natural value (in concentration units) of the ith independent variable, X_i^* is the natural value of the ith independent variable at the centre point of the domain considered and ΔX_i is the step change value of the ith independent variable.

Table 1 – *Ranges and levels of the independent variables (factors) in the CCRD experiments with three factors at five levels each*

Independient variable	Units	Symbols		Coded level of variables				
		uncoded	coded	−1.68	−1	0	1	1.68
[WEICHOL]	g L^{-1}	X_1	x_1	5.00	20.21	42.50	64.79	80.00
[NO$_3^-$]	g L^{-1}	X_2	x_2	3.20	6.04	10.21	14.38	17.22
[PO$_4^{3-}$]	g L^{-1}	X_3	x_3	3.00	6.04	10.50	14.96	18.00

Statistical analysis

The results obtained in the 23 experiments of the experimental design were subjected to multiple regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. These analyses were performed using Essential Regression and Experimental Design for Chemists and Engineers Software (http://www.jowerner.homepage.t-online.de/). Canonical analysis, which was used to predict the stationary point and the shape of the curves generated by the models, was carried out using MATLAB software (http://www.mathworks.com/).

Results and discussion

The experimental central composite rotatable design matrix in the natural (X) and coded (x) levels of variables and the observed experimental data for biomass and PHA production are shown in Table 2. The concentrations of the remaining culture medium components were kept constant as in the minimal basal medium.

An empirical second-order polynomial model for three factors was used to fit the data:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response used as a dependent variable; x_i and x_j the coded levels of independent variables; β_0 the offset term or intercept; β_i the linear terms coefficients; β_{ii} the quadratic terms coefficients and β_{ij} the interaction terms coefficients.

Response models and validation

Application of the multiple linear regression technique to the experimental data provided the following second-order polynomic regression models for biomass production (Y_1) Eq.3 and PHA production (Y_2) Eq.4:

Table 2 – *CCRD matrix of three factors and observed response experimental data*

Exp n°	Variable coded values			Variable natural values			Response experimental values	
	x_1	x_2	x_3	X_1 WEICHOL (g L^{-1})	X_2 NO$_3^{-}$ (g L^{-1})	X_3 PO$_4^{3-}$ (g L^{-1})	Y_1 Biomass (g L^{-1})	Y_2 PHA (g L^{-1})
1	1	1	−1	64.79	14.38	6.04	18.43	4.14
2	−1	−1	−1	20.21	6.04	6.04	7.77	0.88
3	0	0	0	42.50	10.21	10.50	16.02	3.38
4	0	0	0	42.50	10.21	10.50	16.02	3.54
5	0	1.68	0	42.50	17.22	10.50	15.37	2.05
6	−1	1	−1	20.21	14.38	6.04	8.89	0.50
7	0	0	0	42.50	10.21	10.50	15.92	3.40
8	1	1	1	64.79	14.38	14.96	17.68	2.84
9	−1.68	0	0	5.00	10.21	10.50	3.57	0.07
10	0	0	0	42.50	10.21	10.50	15.96	3.14
11	−1	−1	1	20.21	6.04	14.96	8.12	1.25
12	0	−1.68	0	42.50	3.20	10.50	8.35	1.47
13	1.68	0	0	80.00	10.21	10.50	14.41	3.38
14	0	0	1.68	42.50	10.21	18.00	14.64	2.24
15	1	−1	−1	64.79	6.04	6.04	10.94	3.06
16	−1	1	1	20.18	14.38	14.96	8.89	0.65
17	0	0	−1.68	42.50	10.21	3.00	14.05	2.93
18	1	−1	1	64.79	6.04	14.96	11.54	1.83
19	0	0	0	42.50	10.21	10.50	16.37	3.13
20	0	0	0	42.50	10.21	10.50	15.97	3.07
21	0	0	0	42.50	10.21	10.50	15.95	3.35
22	0	0	0	42.50	10.21	10.50	15.50	3.18
23	0	0	0	42.50	10.21	10.50	16.36	3.02

$$Y_1 = 16.01 + 3.16x_1 - 2.47x_1^2 + 2.00x_2 - \\ -1.45x_2^2 + 1.47x_1x_2 - 0.58x_3^2 - 0.21x_2x_3 \tag{3}$$

$$Y_2 = 3.25 + 1.04x_1 - 0.55x_1^2 - 0.54x_2^2 + 0.38x_1x_2 - \\ -0.38x_1x_3 - 0.24x_3^2 - 0.23x_3 + 0.15x_2 \tag{4}$$

Note that a variable selection technique was used to find the "best" model for both responses, including only the significant terms. Among variable selection techniques, Essential Regression incorporates the "stepwise regression" method, which combines both the "forward" and "backward" techniques. Therefore, both polynomial models only consider the significant terms. Moreover, Essential Regression software provides the parameter estimated in order of importance; i.e. the first parameter β is always the coefficient that multiplies the factor with the stronger effect.

An F-test (ANOVA) checked the statistical significance of the second-order model equations. Tables 3a and 3b represent the ANOVA for both models which show that the lack of fit of the regression models is not significant, while Fisher's F-test demonstrates the high significance ($p < 0.05$) of the regression models. Moreover, the R^2 of the biomass and PHA models was calculated to be 0.9971 and 0.9892, respectively, indicating that 99 % and 98 % of the variability in the responses can be explained by the second-order polynomial prediction equations (Eq. 3 and Eq. 4).

The fit of both models is indicated not only by the high R^2 values but also by the data shown in Figs. 1a and 1b. Predicted values of the responses Y_1 and Y_2 were plotted *versus* the experimental values obtained in the experiments performed to obtain the regression models. As shown in the figures, all the points are very close to the diagonal lines and there are no tendencies in the fit of both models. It can be accepted therefore that the models adequately explain the experimental range studied.

Canonical analysis

A second order model with square terms describes a variety of shaped response surfaces. The stationary point of the response surface can be a maximum, a minimum or a saddle point (minimax). It is difficult to understand the surface shape by the mere inspection of the algebraic expression of the model. Moreover, when there are many independent variables in the model, it is also difficult to evaluate the shape of the surface by looking at three-dimensional response surface plots or at isocontour projections of the variables two at a time.

A canonical analysis facilitates the interpretation of the results obtained in the response surface methodology, because it enables analysis of the systems of maxima and minima in many dimensions and, in particular to identification of complicated ridge systems, where direct geometric representation is not possible.[24,26]

Table 3 – *(a) Analysis of Variance (ANOVA) for the significance of the regression model for biomass production (Eq. 3).*
(b) Analysis of Variance (ANOVA) for the significance of the regression model for PHA production (Eq. 4).

(a)

Source	Sum of squares	Degrees of freedom	Mean square	F_0	Probability $P = P:(H_0:F_0 \le F_{crit})$
Regression Model	343	7	47	748.020	6.412 x10^{-18}
Residual of error	0.983	15	0.066		
LOF Error	0.278	6	0.046	0.593	0.730
Pure Error	0.705	9	0.078		
Total	343.983	22			

$R = 0.9986$, $R^2 = 0.9971$, Adjusted $R^2 = 0.9958$, Coefficient of variation = 1.9192

(b)

Source	Sum of squares	Degrees of freedom	Mean square	F_0	Probability $P = P:(H_0:F_0 \le F_{crit})$
Regression Model	28.300	8	3.538	160.25	2.002 x 10^{-12}
Residual of error	0.309	14	0.022		
LOF Error	0.062	6	0.010	0.332	0.902
Pure Error	0.247	8	0.031		
Total	28.610	22			

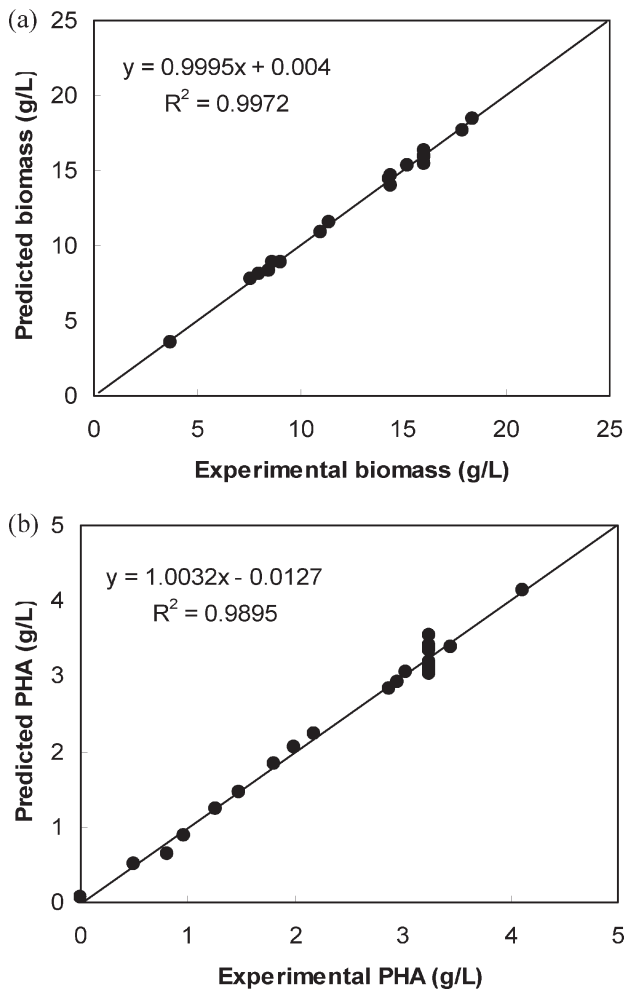$R = 0.9946$, $R^2 = 0.9892$, Adjusted $R^2 = 0.9830$, Coefficient of variation = 6.0473

Fig. 1 – *Comparison between experimental and predicted values for (a) biomass production (Eq. 3) and (b) PHA production (Eq.4).*

Canonical analysis for biomass production

The procedure for the application of the canonical analysis is as follows:[24,25]

1. The coordinates of the stationary point on the response surface, x_s, are determined, which, for biomass, is:

$$x_s = [1, 1.21, -0.23]^T \quad (5)$$

This stationary point is within the explored experimental domain (1.68, –1.68), so the principal axes of the surface are rotated and translated and the canonical coordinate system has its origin at the stationary point. In this case, both cross-product and linear terms are removed from the regression equation. This is called RT canonical analysis.

2. The old variables are transformed into the new ones by using the eigenvectors:

$$w_1 = -0.89\, x_1 + 0.46\, x_2 - 0.02\, x_3 \quad (6)$$

$$w_2 = -0.46\, x_1 - 0.87\, x_2 - 0.19\, x_3 \quad (7)$$

$$w_3 = -0.07\, x_1 - 0.18\, x_2 + 0.98\, x_3 \quad (8)$$

The new variables, w_i, are orientated in the direction of the new axes RT_1, RT_2 and RT_3 and are independent from each other.

It can be observed from Eqs. 6 to 8, that the highest component of the first eigenvector is the first one (–0.89) and, therefore, the new axis RT_1 has a similar orientation to factor x_1, which means that the new variable w_1 can be assimilated with the carbon source (x_1).

Following the same reasoning we can easily deduce that w_2 and w_3 represent nitrogen (x_2) and phosphorus (x_3) sources, respectively.

3. The canonical model for biomass production is established:

$$Y_1 = 18.80 - 2.86w_1^2 - 1.09w_2^2 - 0.56w_3^2 \quad (9)$$

where w_1, w_2 and w_3 are the new variables obtained by the application of the canonical analysis, the independent term is the value of the response at the stationary point and the coefficients are the eigenvalues. It can be observed that the canonical model contains only quadratic terms and, since w_i^2 cannot be negative, it is seen that the shape of the response surface is determined by the sign and the magnitude of the coefficients. In this case, all of them are negative so the response surface has a maximum which corresponds to the value of the response at the stationary point (origin of the canonical coordinate system): i.e. the maximum biomass production is 18.80 g L^{-1}.

Transforming the coordinates of the stationary point, expressed in coded values, into the natural ones we obtain the concentrations of the three main components of the growth media which provide the maximum biomass production:

Carbon source, $X_1 = 64.79$ g L^{-1}

Nitrogen source, $X_2 = 15.25$ g L^{-1}

Phosphorus source, $X_3 = 9.49$ g L^{-1}

As expected, biomass production increased when high concentrations of carbon and nitrogen sources were used in the culture medium. In the light of the above, we can conclude that the use of an appropriate experimental design, together with RSM and canonical analysis, allowed us to improve biomass production over the levels obtained in previous works (4.21 g L^{-1}) more than 4-fold.[11]

4. Analyze of the canonical model either by studying the canonical model or plotting the evolution of the response (Y_i) with the variables (w_i) along the new axis (RT_i).

The larger the absolute value of an eigenvalue, the more pronounced the curvature of the response surface in the associated direction. The canonical equation (Eq. 9) shows that biomass production

varies strongly along the RT_1 axis, while it decreases slowly when the experimental conditions are varied along the RT_3 axis around the stationary point. An intermediate behavior is observed along the RT_2 axis.

If Eq. 9 is reduced to the following equations:

$$Y_1 = 18.80 - 2.86 w_1^2 \qquad (10)$$

$$Y_1 = 18.80 - 1.09 w_2^2 \qquad (11)$$

$$Y_1 = 18.80 - 0.56 w_3^2 \qquad (12)$$

and if these are plotted graphically (Fig. 2), the above mentioned evolution of the response along the new axis, can be observed.
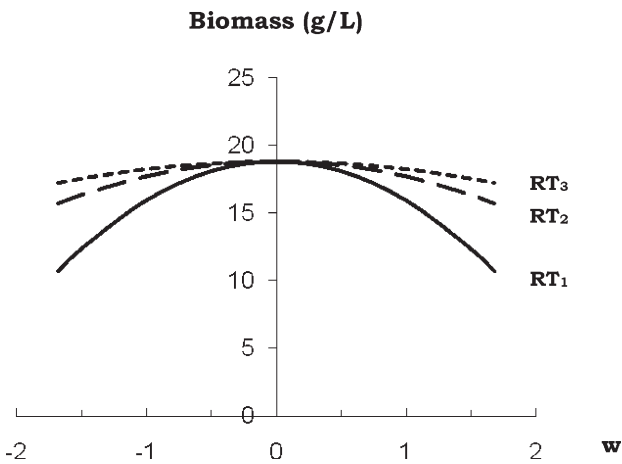
Biomass (g/L)

F i g . 2 – *Curvature of the biomass production (Y_1) along the reference axis RT_i*

In the light of the above, it can be concluded that the variable which exerts the greatest influence on biomass production is the carbon source, followed by the nitrogen source, while the phosphorus concentration has not significant effect on *Pseudomonas aeruginosa* 42A2 growth.

Canonical analysis for PHA production

The procedure is similar to that described previously for biomass production, and so we shall only explain the relevant differences between both procedures.

First, the coordinates for the stationary point of the response surface for PHA production are:

$$Xs = [1.91, 0.83, -1.96]^T \qquad (13)$$

which, in natural variables, are:

Carbon source, $X_1 = 85.30$ g L^{-1}

Nitrogen source, $X_2 = 13.67$ g L^{-1}

Phosphorus source, $X_3 = 1.76$ g L^{-1}

As can be seen, this stationary point is clearly outside the explored domain. In this situation, the canonical analysis consists simply of one rotation of the coordinate system (without translation). This removes only the cross-product terms from the regression model, while the initial origin at the center point is maintained. This is the so-called R canonical analysis.

The variable transformation using the eigenvectors give:

$$Z_1 = 0.75 \, x_1 - 0.61 \, x_2 + 0.27 \, x_3 \qquad (14)$$

$$Z_2 = -0.45 \, x_1 - 0.76 \, x_2 - 0.47 \, x_3 \qquad (15)$$

$$Z_3 = -0.49 \, x_1 - 0.23 \, x_2 + 0.84 \, x_3 \qquad (16)$$

As in the case of biomass production, the new variables z_i, are orientated in the direction of the new axes R_1, R_2 and R_3 and are mutually independent. Taking into account the components of the eigenvectors, it is clear that z_1 can be regarded as equivalent of the carbon source (x_1), z_2 of the nitrogen source (x_2) and z_3 of the phosphorus source (x_3).

The vector of the coefficients of the linear terms is:

$$[0.62, -0.47, -0.74]^T \qquad (17)$$

The response surface model for PHA production is converted into its canonical form to give:

$$Y_2 = 3.25 + 0.62 \, z_1 - 0.47 \, z_2 - 0.74 \, z_3 - 0.77 \, z_1^2 - 0.42 \, z_2^2 - 0.13 \, z_3^2 \qquad (18)$$

where the negative eigenvalues indicate the existence of a maximum.

At first sight, this may not seem a simplification of the response surface model. However, this type of model is very helpful for exploring ridge systems, in which the curvatures are weak along the R_i axes corresponding to eigenvalues close to zero and the variation of the response is largely described by the linear terms of the canonical variables (in our case, z_3).

The coordinates of the stationary point with respect to the new reference system, R are:

$$Z_s = [0.4, -0.55, -2.78]^T \qquad (19)$$

Because the stationary point is outside the experimental domain, the maximum value of the response is not a reliable value since it is obtained by extrapolation beyond the explored domain. The predicted PHA production was, however, promising (4.52 g L^{-1}) and this very high value could be obtained using a culture medium containing high concentrations of all nutrients (mainly carbon and nitrogen sources) and by depletion of phosphate, i.e.: using an unbalanced medium. This result agrees with those find in the bibliography.[2,3,6,7]

Reducing Eq. 18 gives:

$$Y_2 = 3.25 + 0.62 \, z_1 - 0.77 \, z_1^2 \qquad (20)$$

$$Y_2 = 3.25 - 0.47 \, z_2 - 0.42 \, z_2^2 \qquad (21)$$

$$Y_2 = 3.25 - 0.74 \, z_3 - 0.13 \, z_3^2 \qquad (22)$$

which are represented in Fig. 3. Following the same analysis as above, it can be concluded that carbon and phosphorus sources are the factors which exert the greatest influences on the response, although the effect of the nitrogen should not be disregarded. The effect of phosphate concentration on the response is mainly linear, as can be observed by the high value of the corresponding coefficient in Eq. 22.
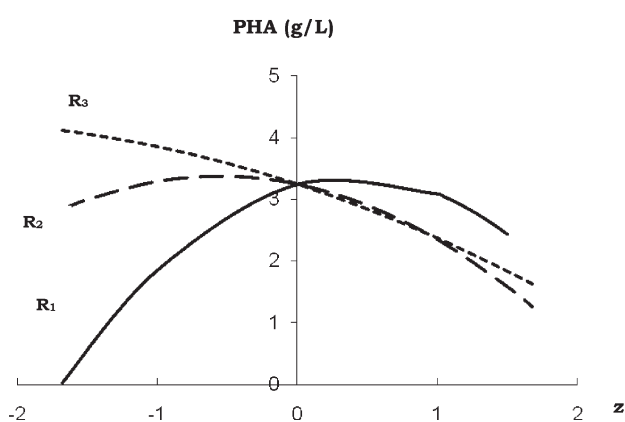

Fig. 3 – *Curvature of the PHA production (Y_2) along the reference axis R_i.*

Validation of the model

It has been reported that the critical evaluation of the importance of a given mathematical model should be made through a series of experiments independent of the one used to obtain the regression model.[27] The regression models obtained for biomass and PHA production by *Pseudomonas aeruginosa* 42A2 were first verified with a set of 10 new experiments carried out for every response by choosing random combinations (distributed within the experimental region). Tables 4a and 4b show the coded and actual levels of the factors used for the validation studies. Figs. 4a and 4b show that, for both biomass and PHA production, the experimental points are uniformly distributed along a straight line, giving regression coefficients of $R^2 = 0.9872$ for biomass and $R^2 = 0.9857$ for PHA production. The consistency between the predicted and experimental values verifies the validity of the model.

Furthermore, to validate the model's capacity for predicting maximum biomass and PHA production, three independent shake flask studies were carried out for every response under optimum conditions for the culture media. The experiments were

Table 4 – *Experimental matrixes for the validation of the regression models (a) biomass production and (b) PHA production by P. aeruginosa 42A2*

(a)

Coded values			Natural values (g/L)		
x_1	x_2	x_3	X_1	X_2	X_3
−1.5	0	0	9.06	10.21	10.50
1	-1.68	0	64.79	3.20	10.50
−1	−1	0	20.21	6.04	10.50
−1	0	0	20.21	10.21	10.50
0	−1	−1	42.50	6.04	6.04
1	0	−1.68	64.79	10.21	3.00
1	1	−1.68	64.79	14.38	3.00
1	1	0	64.79	14.38	10.50
−1.68	0	1	5.00	10.21	14.96
1	0	0	64.79	10.21	10.50

(b)

Coded values			Natural values (g/L)		
x_1	x_2	x_3	X_1	X_2	X_3
0	1	−1.68	42.50	14.38	3.00
−1	1	0	20.21	14.38	10.50
0	−1	0	42.50	6.04	10.50
1	0	0	64.79	10.21	10.50
1.68	1.68	−1	80.00	17.22	6.04
1	0	1	64.79	10.21	14.96
1	1.68	−1	64.79	17.22	6.04
0	−1	1.68	42.50	6.04	18.00
1	1	0	64.79	14.38	10.50
−1.68	0	1	5.00	10.21	14.96

carried out using the values of the stationary points calculated in the canonical analysis (i.e., carbon, nitrogen and phosphorus source concentrations).

The three replicate experiments for biomass production yielded an average maximum concentration of 18.73 g L^{-1}, which is close to the predicted value (18.80 g L^{-1}). The three replicate experiments for PHA production yielded an average maximum concentration of 4.63 g L^{-1} and the predicted response, using the concentrations specified by canonical analysis of the response surface of the PHA production model, was 4.52 g L^{-1}.

Conclusions

Experimental results showed that canonical analysis of the response surfaces was an efficient method for optimizing the components of the medium and for increasing biomass and PHA production from *Pseudomonas aeruginosa* 42A2 when an industrial oil subproduct was used as a cheap carbon source.
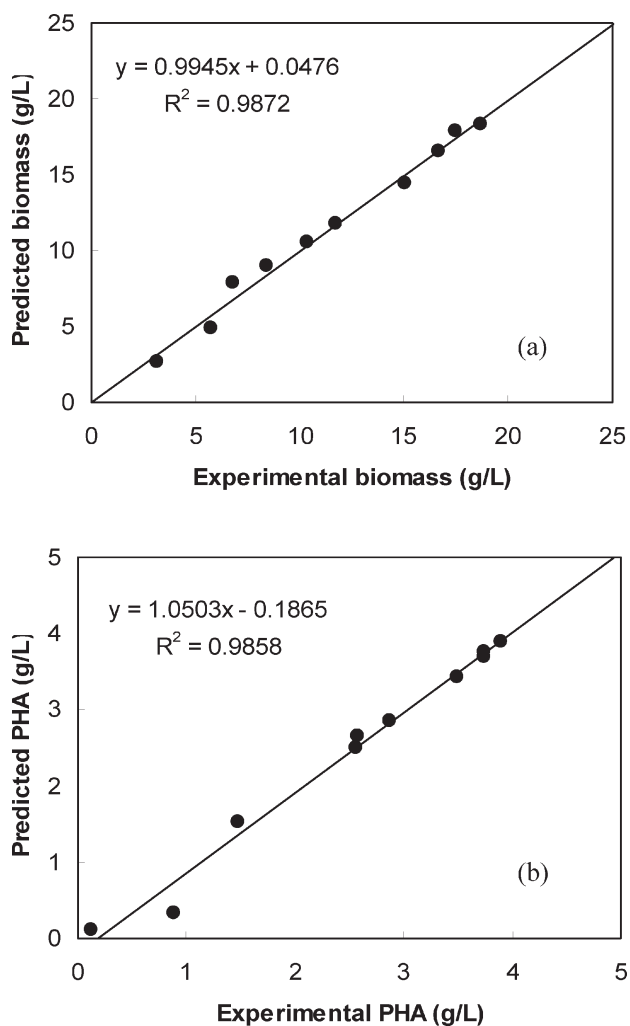
F i g . 4 – *Models validation. Comparison between experimental and predicted values for (a) biomass and (b) PHA production.*

We conclude from our results that biomass production mainly depends on the concentration of the carbon source and nitrate. Theoretically, the maximum amount of biomass produced by *Pseudomonas aeruginosa* 42A2 was predicted to be 18.80 g L^{-1} (4.5-fold increase) when the optimized composition of the growth medium was as follows: 64.79 g L^{-1}, WEICHOL; 20.90 g L^{-1}, $NaNO_3$ (15.25 g NO_3^-/L) and 10.61 g L^{-1}, K_2HPO_4/5.04 g L^{-1} KH_2PO_4 (9.49 g PO_4^{3-}/L). The empirical value obtained was 18.73 g L^{-1} of biomass.

We found that PHA production depends on the concentration of the three constituents of the medium but for maximum production high levels of carbon and nitrogen sources and very low concentration of phosphorus were required. The final composition of the defined medium to maximize PHA production after optimization was as follows: 85.30 g L^{-1}, WEICHOL; 18.73 g L^{-1}, $NaNO_3$ (13.67 g NO_3^-/L) and 1.97 g L^{-1}, K_2HPO_4/0.98 g L^{-1} KH_2PO_4 (1.76 g PO_4^{3-}/L). Theoretically, this medium produces 4.52 g L^{-1} of PHA and the actual value obtained was 4.63 g L^{-1} of PHA.

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