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Utilization of response surface methodology to optimize the culture media for the production of rhamnolipids by *Pseudomonas aeruginosa* AT10

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Abstract: ***Pseudomonas aeruginosa*** **AT10 produced a mixture of surface-active rhamnolipids when cultivated on mineral medium with waste free fatty acids as carbon source. The development of the production process to an industrial scale included the design of the culture medium. A 2^4 full factorial, central composite rotational design and response surface modelling method (RSM) was used to enhance rhamnolipid production by** ***Pseudomonas aeruginosa*** **AT10. The components that are critical for the process medium were the carbon source, the nitrogen source ($NaNO_3$), the phosphate content (K_2HPO_4/KH_2PO_4 2:1) and the iron content ($FeSO_4 \cdot 7H_2O$). Two responses were measured, biomass and rhamnolipid production. The maximum biomass obtained was 12.06 g dm^{-3} DCW, when the medium contained 50 g dm^{-3} carbon source, 9 g dm^{-3} $NaNO_3$, 7 g dm^{-3} phosphate and 13.7 mg dm^{-3} $FeSO_4 \cdot 7H_2O$. The maximum concentration of rhamnolipid, 18.7 g dm^{-3}, was attained in medium that contained 50 g dm^{-3} carbon source, 4.6 g dm^{-3} $NaNO_3$, 1 g dm^{-3} phosphate and 7.4 mg dm^{-3} $FeSO_4 \cdot 7H_2O$.**

Keywords: response surface methodology (RSM); medium design; biosurfactants; rhamnolipids; *Pseudomonas aeruginosa*

INTRODUCTION

Biosurfactants are amphipathic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi, and include peptides, glycolipids, lipopeptides, fatty acids and phospholipids. These molecules have tremendous potential for applications in the petrochemical, pharmaceutical, cosmetics and food industries as emulsifiers and de-emulsifiers, since they are biodegradable and less toxic than synthetic surfactants.[1–3]

The most commonly isolated and best-studied biosurfactants are glycolipids and phospholipids.[4] Rhamnolipids are a class of compound containing one or two 3-hydroxy fatty acids of various chain length (C_8–C_{22}) esters linked to a mono- or di-rhamnose moiety and produced as a complex mixture by a species of *Pseudomonas*.[3,5] The biosynthetic pathway for rhamnolipid was proposed by Burger *et al*[6] as sequential glycosyl transfer reactions, each catalysed by a specific rhamnosyltransferase.

Pseudomonas aeruginosa AT10 produces a mixture of up to seven rhamnolipid homologues when cultivated in a mineral medium with waste free fatty acids (WFFA) from refined-soybean oil as carbon source.[7] This rhamnolipid mixture decreases the surface tension of the culture medium from 55 to 26.8 mN m^{-1}, at a critical micelle concentration (cmc) of 150 mg dm^{-3}. Before rhamnolipids are produced on an industrial scale the process parameters must be optimized. Ways to enhance the yield include (a) strain improvement, (b) medium development, (c) process optimization and (d) the use of alternative, inexpensive substrates.[8–12]

The conventional method for medium optimization involves changing one variable at a time, keeping the other factors fixed at a specific set of conditions. This method may lead to unreliable results and wrong conclusions. Moreover, carrying out experiments with every possible combination of the variables is impractical, because of the large number of experiments required.[13]

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Response surface methodology (RSM), which includes factorial designs and regression analysis, can better deal with multifactor experiments. RSM is a collection of statistical techniques for designing experiments, building models, and evaluating the effects of factors. The desirable responses define the optimization of a factor for desirable responses.[14–20]

The aim of this work is the optimization of the culture medium for rhamnolipid production by *Pseudomonas aeruginosa* AT10. Two responses have been measured: biomass ($\boldsymbol{Y}_1$) and rhamnolipid ($\boldsymbol{Y}_2$) concentrations, and the factors were: carbon source (WFFA), nitrogen source ($NaNO_3$); phosphate (K_2HPO_4/KH_2PO_4 ratio 2:1); and iron ($FeSO_4 \cdot 7H_2O$).

MATERIAL AND METHODS

Microorganism

Pseudomonas aeruginosa AT10, was isolated from contaminated soils of the ERASOL food-oil refinery in Santiago de Cuba (Cuba). The strain was grown at 30 °C for 24 h and maintained at 4 °C on TSA plates and alternatively on cryo-billes (Combourg, France) at −20 °C.

Media composition

The medium for inoculum preparation and production contained the following mineral salts ($g\,dm^{-3}$): $CaCl_2$, 0.01; KCl, 0.10; $MgSO_4 \cdot 7H_2O$, 0.50; yeast extract, 0.10 and was supplemented with 0.05 cm^3 dm^{-3} of a trace elements solution containing ($g\,dm^{-3}$): H_3BO_3, 0.26; $CuSO_4 \cdot 5H_2O$, 0.50; $MnSO_4 \cdot 7H_2O$, 0.50; $MoNa_2O_4 \cdot 2H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.70. The concentrations of the carbon source, $NaNO_3$, K_2HPO_4/KH_2PO_4 (ratio 2:1) and $FeSO_4 \cdot 7H_2O$ were varied according to the experimental design. The levels of the test variables are shown in Table 1. The pH value of the media was adjusted to 6.8.

The carbon source used was WFFA from refined soybean oil with the following composition (w/w): 17% the most volatile fraction ($<C_{12}$), 6.23% palmitic acid, 2.91% stearic acid, 21.1% oleic acid, and 48.4% linoleic acid.

Shake flask experiments

Experiments were carried out in 250 cm^3 baffled Erlenmeyer flasks containing 50 cm^3 of medium. The medium was sterilized for 20 min at 120 °C and 1.01×10^5 Pa. Cultures were incubated for 96 h in a reciprocal shaker (150 rpm) at 30 °C. All the experiments were carried out in triplicate.

Table 1. Actual factor levels corresponding to coded factor levels

		Actual values				
Variable	*Code*	*−2*	*−1*	*0*	*1*	*2*
WFFA ($g\,dm^{-3}$)	X_1	10	20	30	40	50
$NaNO_3$ ($g\,dm^{-3}$)	X_2	1	3	5	7	9
Phosphates ($g\,dm^{-3}$)	X_3	1	2.5	4	5.5	7
$FeSO_4 \cdot 7H_2O$ ($mg\,dm^{-3}$)	X_4	1	6	11	16	21

Phosphates ($g\,dm^{-3}$) = K_2HPO_4/KH_2PO_4 (ratio 2:1).

Analytical methods

Rhamnolipid concentration was quantified by a colorimetric method as rhamnose content using a rhamnose standard.[21] Rhamnolipid content was calculated by multiplying rhamnose concentration by a factor of 3. This factor was calculated experimentally using the calibration curve of rhamnose (correlation rhamnolipid/rhamnose). Cellular biomass was determined by measuring the dry weight of the cells from 5 dm^3 of sample after desiccation at 100 °C for 24 h.

Experimental design

A 2^4 full-factorial central composite design for the four test variables (factors) selected, each one at five levels (concentrations), was employed to fit a regression model. This procedure required 31 experiments. All the experiments were carried out in triplicate.

The dependent variables selected for this study (responses) were: biomass concentration (g dry weight dm^{-3}), $\boldsymbol{Y}_1$, and rhamnolipid concentration ($g\,dm^{-3}$), $\boldsymbol{Y}_2$. We determined the coded factor levels as follows: $X_1 = (\text{carbon source} - 30)/10$; $X_2 = (\text{nitrogen} - 5)/2$; $X_3 = (\text{phosphorus} - 4)/1.5$ and $X_4 = (\text{iron} - 11)/5$. Table 1 contains the actual factor levels corresponding to coded factor levels.

Statistical analysis

Data from the factorial 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 Software. Microsoft Excel 7.0 (Microsoft Corp, Redmont, WA) software was used to optimize the composition of the culture medium and to plot the experimental data and models. All data presented are mean values of three determinations.

RESULTS AND DISCUSSION

Regression models

When *P aeruginosa* AT10 was grown in a mineral medium with WFFA, rhamnolipid production was 3.6 g dm^{-3}. The biomass obtained was 4.23 g dm^{-3}.[7]

Previous studies on rhamnolipid production[22] indicated that four experimental variables influenced rhamnolipid accumulation: the carbon source, the nitrogen source, the phosphate source and iron (Table 1).

The design matrix of the variables in both coded and natural units along with the responses values are presented in Table 2. An empirical second-order polynomial model was used to fit the data.[17] For four

Table 2. Central composite design matrix of four factors in coded and natural units along with the responses

	Coded values				*Actual values*				*Responses (g dm^{-3})*	
	X_1	X_2	X_3	X_4	X_1 (g dm^{-3})	X_2 (g dm^{-3})	X_3 (g dm^{-3})	X_4 (mg dm^{-3})	Biomass (Y_1)	Rhamnolipids (Y_2)
1	−1	−1	−1	−1	20	3	2.5	6	3.98	2.73
2	1	−1	−1	−1	40	3	2.5	6	4.50	9.39
3	−1	1	−1	−1	20	7	2.5	6	3.91	2.55
4	1	1	1	1	40	7	2.5	6	7.80	8.67
5	−1	−1	1	−1	20	3	5.5	6	3.00	1.59
6	1	−1	1	−1	40	3	5.5	6	5.11	6.69
7	−1	1	1	−1	20	7	5.5	6	3.76	1.20
8	1	1	1	−1	40	7	5.5	6	8.33	5.88
9	−1	−1	−1	1	20	3	2.5	16	4.50	3.37
10	1	−1	−1	1	40	3	2.5	16	5.00	9.69
11	−1	1	−1	1	20	7	2.5	16	4.56	1.41
12	1	1	−1	1	40	7	2.5	16	8.23	6.93
13	−1	−1	1	1	20	3	5.5	16	4.14	2.91
14	1	−1	1	1	40	3	5.5	16	5.62	7.74
15	−1	1	1	1	20	7	5.5	16	4.83	0.84
16	1	1	1	1	40	7	5.5	16	8.63	4.71
17	−2	0	0	0	10	5	4	11	1.63	2.40
18	2	0	0	0	50	5	4	11	5.97	14.58
19	0	−2	0	0	30	1	4	11	3.35	2.79
20	0	2	0	0	30	9	4	11	6.38	0.30
21	0	0	−2	0	30	5	1	11	6.69	7.41
22	0	0	2	0	30	5	7	11	6.99	3.93
23	0	0	0	−2	30	5	4	1	3.93	3.90
24	0	0	0	2	30	5	4	21	5.26	4.14
25	0	0	0	0	30	5	4	11	6.25	5.04
26	0	0	0	0	30	5	4	11	5.90	4.98
27	0	0	0	0	30	5	4	11	6.00	4.98
28	0	0	0	0	30	5	4	11	6.61	4.92
29	0	0	0	0	30	5	4	11	5.13	4.62
30	0	0	0	0	30	5	4	11	6.05	5.28
31	0	0	0	0	30	5	4	11	6.13	4.95

X_1 = WFFA g dm^{-3}, X_2 = $NaNO_3$ g dm^{-3}, X_3 = phosphates g dm^{-3}, X_4 = $FeSO_4 \cdot 7H_2O$ mg dm^{-3}.

factors, the quadratic model takes the following form:

$$\begin{aligned} \boldsymbol{Y} = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 \\ & + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 \\ & + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 \\ & + b_{24}X_2X_4 + b_{34}X_3X_4 \end{aligned} \quad (1)$$

where b_0 is the value of the fixed response at the central point of the experiment, which is the point (0, 0, 0, 0); b_1, b_2, b_3 and b_4 are the coefficients of the linear terms; b_{11}, b_{22}, b_{33} and b_{44} are the coefficients of the quadratic terms and b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are the coefficients of the cross products.

Tables 3a and 3b show the results of the quadratic response-surface model fitting for both responses $\boldsymbol{Y}_1$ and $\boldsymbol{Y}_2$, respectively, in the form of Analysis of Variance (ANOVA).

ANOVA is commonly used to summarize the test for significance and adequacy of the model. In computer programs the significance level (also called error probability, P) is given in addition to the Fisher variance ratio. The greater the F values are from unity,

Table 3a. Analysis of variance (ANOVA) for the quadratic model (eqn (1)) for biomass (response variable Y_1)

Source	*Sum of squares*	*Degrees of freedom*	*Mean square*	F	*Probability* P (>F)
Regression	77.44	14	5.53	36.18	1.97e^{-09}
Residual	2.446	16	0.153		
LOF Error	1.226	10	0.123	0.603	0.772
Pure Error	1.220	6	0.203		
Total	79.88	30			

R = 0.9846, R^2 = 0.9694, adjusted R^2 = 0.9426, coefficient of variation = 7.207%.

Source	*Sum of squares*	*Degrees of freedom*	*Mean square*	F	*Probability* P (>F)
Regression	276.86	14	19.78	252.75	$4.94e^{-16}$
Residual	1.252	16	0.07824		
LOF Error	1.025	10	0.103	2.7156	0.117
Pure Error	0.227	6	0.03776		
Total	278.11	30			

$R = 0.9977$, $R^2 = 0.9955$, adjusted $R^2 = 0.9916$, coefficient of variation = 5.761%.

Table 3b. Analysis of variance (ANOVA) for the quadratic model (eqn (1)) for rhamnolipids (response variable Y_2)

the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are true. On the other hand, a small value for the computed error probability indicates that the regression model is more significant.

The ANOVA given in Tables 3a and 3b also shows the test for lack of fit. If replicate measurements are present in the experimental matrix, ie responses based on the same settings for the independent variables, which is the case here, a test can be performed that gives the significance of the replicate error in comparison to the model dependent error. In other words, the test splits the Residual into a contribution from the pure error, which is based on the replicate measurements, and a fraction which is due to the lack of fit based on the model performance. The statistical test for lack of fit is similar to the *F*-test for significance of the model described above. If the *F* value is large and the *P* value is small, the lack of fit error is significant, ie there might be a contribution in the regression–response relationship not accounted for by the model.

Tables 3a and 3b show that both models were significant (adjusted $R^2 = 0.9426$ for biomass and adjusted $R^2 = 0.9916$ for rhamnolipid). However, the lack of fit for rhamnolipid production was significant ($P = 0.117$). This suggests that the model does not accurately represent data in the experimental region. This indicates that higher-order terms might have to be included in the regression model. Since each factor has five levels, up to quadratic terms could be included in the model. Therefore, a variable selection technique was used to find a better model for both responses: biomass and rhamnolipid production. Among variable selection techniques, Essential Regression incorporates the 'stepwise regression' method, which combines both the 'forward' and 'backward' techniques for the selection of the 'best' model.

The functional forms of the new models for both responses are as follows:

$$\boldsymbol{Y}_1 = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}{X_1}^2 + b_{33}{X_3}^2 + b_4X_4 + b_{44}{X_4}^2 + b_{22}{X_2}^2 + b_{13}X_1X_3 \quad (2)$$

$$\boldsymbol{Y}_2 = b_0 + b_1X_1 + b_{22}{X_2}^2 + b_{11}{X_1}^2 + b_3X_3 + b_2X_2 + b_{24}X_2X_4 + b_{13}X_1X_3 + b_{44}{X_4}^2 + b_{122}X_1{X_2}^2 + b_{33}{X_3}^2 + b_{34}X_3X_4 + b_{12}X_1X_2 + b_{14}X_1X_4 \quad (3)$$

Tables 4a and 4b show the analysis of variance for both new models. It can be observed that, in the case of the first response variable, $\boldsymbol{Y}_1$, only an irrelevant improvement of the fitting has been obtained with the application of the stepwise method. The new model (eqn (2)) has a larger adjusted R^2 value

Source	*Sum of squares*	*Degrees of freedom*	*Mean square*	F	*Probability* P (>F)
Regression	77.03	9	8.559	63.00	$3.576e^{-13}$
Residual	2.853	21	0.136		
LOF Error	1.588	14	0.113	0.6274	0.783
Pure Error	1.265	7	0.181		
Total	79.88	30			

$R = 0.9820$, $R^2 = 0.9643$, adjusted $R^2 = 0.9490$, coefficient of variation = 6.794%.

Table 4a. Analysis of variance (ANOVA) for the nine-terms quadratic model (eqn (2)) for biomass (response variable Y_1)

Source	*Sum of squares*	*Degrees of freedom*	*Mean square*	F	*Probability* P (>F)
Regression	277.47	13	21.34	569.68	$9.092e^{-20}$
Residual	0.637	17	0.03747		
LOF Error	0.382	10	0.03816	1.0461	0.492
Pure Error	0.255	7	0.03648		
Total	278.11	30			

$R = 0.9989$, $R^2 = 0.9977$, adjusted $R^2 = 0.9960$, coefficient of variation = 3.986%.

Table 4b. Analysis of variance (ANOVA) for the 13-terms cubic model (eqn (3)) for rhamnolipids (response variable Y_2)

(0.9490 > 0.9426), and a smaller coefficient of variation (6.794 < 7.207), making the lack of fit more insignificant ($P = 0.783 > 0.772$). However, the number of explanatory variables (9) is smaller in the new model (eqn (2)) than in the second-order model (14) (eqn (1)).

In the case of the second response variable, $\boldsymbol{Y}_2$, the third-order model (eqn (3)) is superior to the second-order full model (eqn (1)): it has a larger adjusted R^2 value (0.9960 > 0.9916) and a smaller coefficient of variation (3.986 < 5.761), with the lack of fit being insignificant ($P = 0.492 > 0.117$) and the number of variables being slightly smaller (13 < 14).

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 (dark dots) of the responses $\boldsymbol{Y}_1$ and $\boldsymbol{Y}_2$ were plotted *versus* the experimental values obtained in the experiments performed to obtain the regression models.

The Student t distribution and the corresponding P values, along with the parameter estimated, are shown in Tables 5a and 5b for biomass and rhamnolipid regression models –selected through variable selection. The P values are used to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the independent variables. The lower the magnitude of P, the more significant is the corresponding coefficient.[17] Essential Regression provides the parameter estimate in order of importance; ie the first parameter b_1 is always the coefficient that multiplies the effect with the strongest effect.

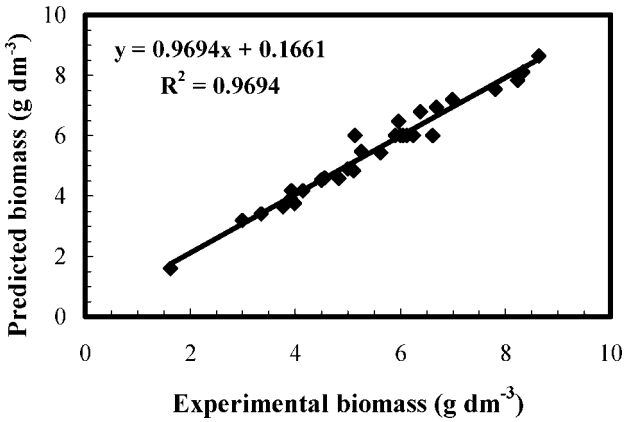

Figure 1a. Theoretical values of biomass predicted by regression model plotted *versus* experimental values.

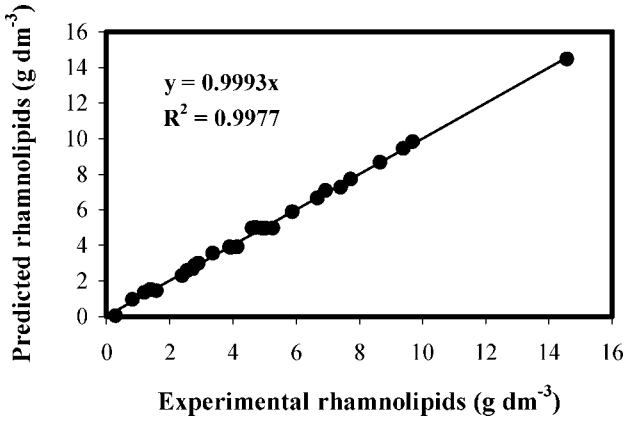

Figure 1b. Theoretical values of rhamnolipids predicted by regression model plotted *versus* experimental values.

Biomass: effects of factors and maximum values of the factors

As expected, the carbon and nitrogen sources had a large influence on biomass production, which was high ($P = 2.47e^{-13}$ and $P = 2.49e^{-10}$ respectively), because these nutrients are essential for bacterial metabolism in protein synthesis and macromolecules[23,24] and the interaction of both ($P = 1.58e^{-07}$). The quadratic effect of phosphate was more pronounced than its interaction with carbon concentration and the linear effect of phosphate was not significant (not included in the regression model). Phosphates are required because of the double function of phosphate as a constitutive element of nucleic acids, phospolipids or cell wall polymers, however little is known about its metabolic interaction.[23–25]

Although iron is a fundamental micronutrient in the cellular metabolism of the oxidative phosphorylation,[23,24] the influence of iron concentration was low in its linear and quadratic terms and the interaction with other effects is not reflected in the model.

Three-dimensional response surface plots have been drawn with the vertical axis representing biomass ($\boldsymbol{Y}_1$) and two horizontal axes representing the coded levels of the two most significant factors for response (X_1 and X_2). The factors not represented by the two horizontal axes were fixed at their optimum levels (Fig 2 and Fig 3). The maximum values of the test variables were obtained by using Microsoft Excel Solver, which is incorporated to the Essential Regression software.

The maximum obtained for biomass ($\boldsymbol{Y}_1$, eqn (2), was (X_1, X_2, X_3, X_4) = (2, 2, 2, 0.55). Recording the coded levels back to the original levels, the following results were obtained: WFFA = 50 g dm^{-3};

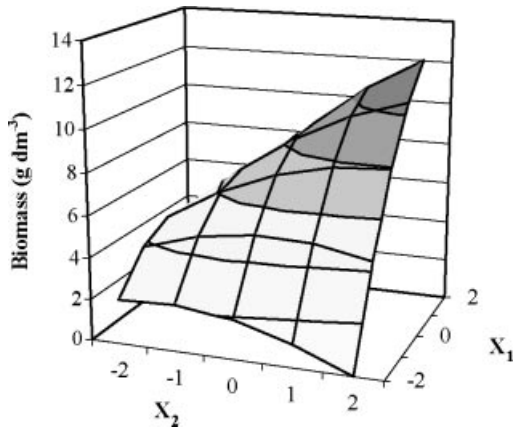

Figure 2. Response surface for the effects of carbon source (X_1) and nitrogen (X_2) concentrations on the growth of *Pseudomonas aeruginosa* AT10 at $X_3 = 2$ and $X_4 = 0.55$ (phosphate = 7 g dm^{-3} and $FeSO_4 \cdot 7H_2O$ = 13.75 mg dm^{-3}).

Table 5a. Coefficient estimates in the regresion model for biomass selected through variable selection

Variable	*Coeficient estimate*	*Standard error*	t *value*	P *value*
Intercept	$b_0 = 6.010$	0.13931	43.142	$5.46e^{-22}$
X_1	$b_1 = 1.218$	0.07523	16.183	$2.47e^{-13}$
X_2	$b_2 = 0.844$	0.07523	11.220	$2.49e^{-10}$
X_1X_2	$b_{12} = 0.708$	0.09214	7.678	$1.58e^{-07}$
X_1^2	$b_{11} = -0.495$	0.06892	−7.185	$4.41e^{-07}$
X_3^2	$b_{33} = 0.265$	0.06892	3.842	$9.48e^{-04}$
X_4	$b_4 = 0.324$	0.07523	4.309	$3.11e^{-04}$
X_4^2	$b_{44} = -0.296$	0.06892	−4.301	$3.16e^{-04}$
X_2^2	$b_{22} = -0.229$	0.06892	−3.322	$3.24e^{-03}$
X_1X_3	$b_{13} = 0.211$	0.09214	2.293	$3.23e^{-02}$

Table 5b. Coefficient estimates in the regresion model for rhamnolipids selected through variable selection

Variable	*Coeficient estimate*	*Standard error*	t *value*	P *value*
Intercept	$b_0 = 4.967$	0.07316	67.89	$3.847e^{-22}$
X_1	$b_1 = 3.045$	0.06844	44.49	$4.876e^{-19}$
X_2^2	$b_{22} = -0.883$	0.03620	−24.38	$1.149e^{-14}$
X_1^2	$b_{11} = 0.854$	0.03620	23.58	$1.992e^{-14}$
X_3	$b_3 = -0.839$	0.03951	−21.24	$1.114e^{-13}$
X_2	$b_2 = -0.704$	0.03951	−17.83	$1.943e^{-12}$
X_2X_4	$b_{24} = -0.483$	0.04839	−9.977	$1.601e^{-08}$
X_1X_3	$b_{13} = -0.383$	0.04839	−7.924	$4.158e^{-07}$
X_4^2	$b_{44} = -0.264$	0.03620	−7.288	$1.267e^{-06}$
$X_1X_2^2$	$b_{122} = -0.352$	0.08382	−4.194	$6.09e^{-04}$
X_3^2	$b_{33} = 0.149$	0.03620	4.108	$7.35e^{-04}$
X_3X_4	$b_{34} = 0.173$	0.04839	3.584	$2.29e^{-03}$
X_1X_2	$b_{12} = -0.170$	0.04839	−3.507	$2.71e^{-03}$
X_1X_4	$b_{14} = -0.127$	0.04839	−2.615	$1.809e^{-02}$

$NaNO_3 = 9\,g\,dm^{-3}$; phosphates $= 7\,g\,dm^{-3}$ and $FeSO_4 \cdot 7H_2O = 13.75\,mg\,dm^{-3}$. In these conditions, the predicted biomass was $12.05\,g\,dm^{-3}$. If eqn (3) is solved using these values of the variables, $2.86\,g\,dm^{-3}$ of rhamnolipid should be produced and the production yield calculated ($Y_{p/x}$) was 0.23. As expected, the medium composition for this response had a balanced composition, which is a necessary condition for high biomass production.[24] Although the maximum response for biomass was obtained (Fig 2), the cellular yield of a carbon source ($Y_{x/C} = 0.24$) was very low, suggesting the system may accept higher levels of carbon source. However the carbon source (WFFA) is a highly hydrophobic and semi-solid waste, the growth of bacterial population may be inhibited by the toxicity or inaccessibility of the substrate at high concentrations.

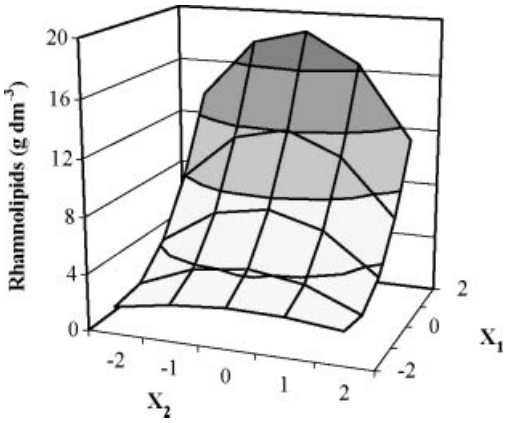

Figure 3. Response surface for the effects of carbon source (X_1) and nitrogen (X_2) concentrations on the production of rhamnolipids by *Pseudomonas aeruginosa* AT10 at $X_3 = -2$ and $X_4 = -0.97$ (phosphate $= 1\,g\,dm^{-3}$ and $FeSO_4 \cdot 7\,H_2O = 7.4\,mg\,dm^{-3}$).

Rhamnolipid: effects of factors and maximum values of the factors

The parameter estimate and the corresponding P values for rhamnolipid production (Table 5b) suggest that carbon source ($P = 4.876e^{-19}$), nitrogen ($P = 1.149e^{-14}$) and phosphate ($P = 1.992e^{-14}$) produce the largest effect on rhamnolipid production. The relationships between carbon source and the other factors are also significant although the influence did not vary. The quadratic term of iron concentration ($P = 1.267e^{-06}$) and its interaction with the other three effects have a weak influence. In short, the regression model described in eqn (3) predicts that all the test variables have a strong effect on rhamnolipid production.

Rhamnolipid synthesis involves two central metabolic pathways, with energy being consumed;[26] an excess of carbon source was required in the medium to carry out the metabolic pathways. Nitrogen is a fundamental macronutrient for cellular metabolism. Moreover, the rhamnolipid overproduction is closely related to nitrogen limitation in the culture med-

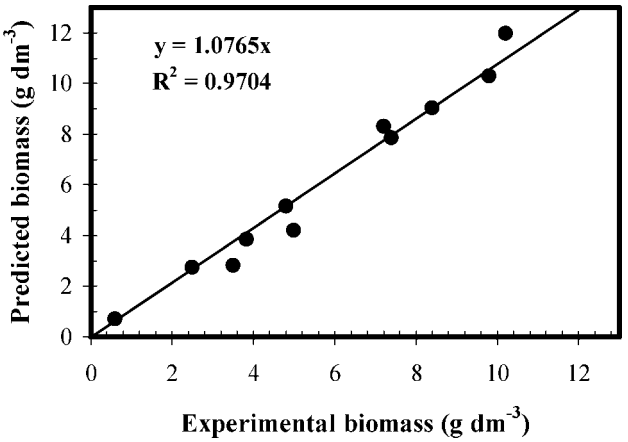

Figure 4a. Experimental values of biomass *versus* theoretical values predicted by regression model (eqn (2)).

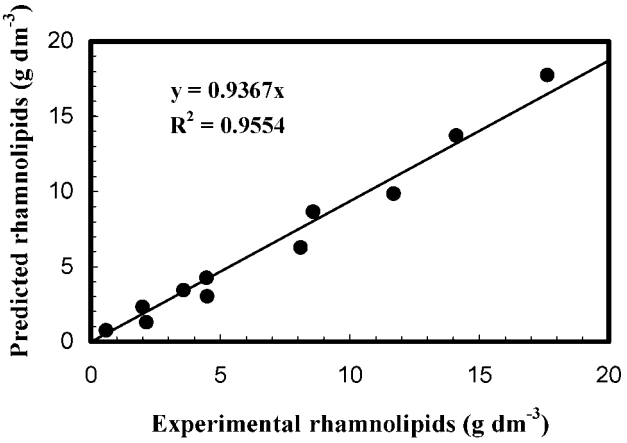

Figure 4b. Experimental values of rhamnolipids *versus* theoretical values predicted by regression model (eqn (3)).

ium.[26,27] When the medium is nitrogen-limited, the protein synthesis is blocked and the cellular metabolism is switched to carbohydrate metabolism synthesis and rhamnolipid production is enhanced.[28] Conversely, an excess of nitrogen source leads to a decrease in the rhamnolipid synthesis, driving the metabolism towards cell growth.

The three-dimensional response surface plots have been drawn with the vertical axis representing rhamnolipid production ($\boldsymbol{Y}_2$) and two horizontal axes representing the coded levels of the two most significant factors for response (X_1 and X_2). Factors not represented by the two horizontal axes are fixed at their optimum levels (Fig 3). The maximum values of the test variables were obtained by using Microsoft Excel Solver, which is included in the Essential Regression software.

The maximum point ($\boldsymbol{Y}_2$, eqn (3) obtained for rhamnolipid production, was (2, −0.18, −2, −0.97). It was equivalent to: WFFA = 50 g dm^{-3}; $NaNO_3$ = 4.64 g dm^{-3}; phosphates = 1 g dm^{-3} and $FeSO_4 \cdot 7H_2O$ = 7.4 mg dm^{-3}. At these conditions, the maximum amount of rhamnolipid produced was 18.66 g dm^{-3} and biomass 5.67 g dm^{-3} with a production yield $Y_{p/x}$ was 3.29 (Fig 3). Frequently, in production processes, different conditions for cell growth and production may be observed, in the case of this study the optimal conditions for biomass ($\boldsymbol{Y}_1$) and rhamnolipid production ($\boldsymbol{Y}_2$) differed. Rhamnolipid production was enhanced when nitrogen and phosphate were limited because in these conditions the protein synthesis was affected, thus limiting biomass formation.

Confirmative studies

It has been reported that the critical evaluation of the importance of a mathematical model should be made by using a series of experiments independent of the ones used to obtain the regression model.[29] In order to check the prediction of the regression models obtained, a set of 11 aleatory experiments was carried out. The experimental results obtained in a separate set of experiments (per triplicate) different from those used to fit the regression models were matched to those predicted by both models. Table 6 shows the coded and actual levels of the factors for confirmative studies: 16.50 g dm^{-3} for rhamnolipids was found whereas that predicted by the model was 18.66 g dm^{-3}; experimental confirmation of 10 g dm^{-3} of biomass was achieved in comparison with 12 g dm^{-3}

Coded values				*Actual values*			
X_1	X_2	X_3	X_4	X_1 (g dm^{-3})	X_2 (g dm^{-3})	X_3 (g dm^{-3})	X_4 (mg dm^{-3})
2	−1	−2	0	50	3	1	11
2	2	2	0	50	9	7	11
2	−2	−2	2	50	1	1	21
0	−2	−2	1	30	1	1	16
0	2	−2	0	30	9	1	11
1	−2	−2	2	40	1	1	21
−1	1	−2	2	20	7	1	21
1	2	−2	2	40	9	1	21
2	2	−2	0	50	9	1	11
−2	2	0	2	10	9	4	21
1	−2	0	1	40	1	4	16
1	2	0	−1	40	9	4	6

Table 6. Coded and actual values of the factors used in the corroborative experiments

predicted by the model. Figures 4a and 4b show that the fit of the experimental and theoretical data to a straight line gave regression coefficients of $R^2 = 0.9704$ for biomass and $R^2 = 0.9554$ for rhamnolipid production.

These results validate (within the interval studied) the technique to design the culture medium for rhamnolipid production, and the responses predicted by the regression models were powerful.

CONCLUSIONS

On the basis of response surface methodology it was possible to select the experimental conditions that lead to maximum biomass and rhamnolipid production. The maximum biomass obtained by *Pseudomonas aeruginosa*. AT10 was $12.06\,g\,dm^{-3}$ when the medium contained $50\,g\,dm^{-3}$ of WFFA; $9\,g\,dm^{-3}$ of $NaNO_3$; $7\,g\,dm^{-3}$ phosphates and $13.7\,mg\,dm^{-3}$ $FeSO_4 \cdot 7H_2O$. However the maximum rhamnolipid production reached was $18.66\,g\,dm^{-3}$ of rhamnolipid with $50\,g\,dm^{-3}$ of WFFA; $4.6\,g\,dm^{-3}$ of $NaNO_3$; $1\,g\,dm^{-3}$ phosphates and $7.4\,mg\,dm^{-3}$ $FeSO_4 \cdot 7H_2O$.

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