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Use of response surface design in the optimization of starter cultures for enhanced rhamnolipid production by *Pseudomonas nitroreducens*

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The development and management of an inoculum through various stages has a definite effect on subsequent performance and economics of a microbial process. To achieve this, application of response surface modeling in the optimization of the primary and secondary inoculum build-up of *Pseudomonas nitroreducens* for enhanced rhamnolipid production was presented in this study. This involved systematic studies to understand the individual, cumulative and mutual interactive effects of the two parameters, inoculum age and size on rhamnolipid production. The optimal combination of factors was as follows: primary inoculum age = 61 h, primary inoculum size = 1% (v/v), secondary inoculum age = 28 h and secondary inoculum size = 20% (v/v). The model predicted a maximum rhamnolipid yield of 7.944 g/L. This was as a result of strong interaction between secondary inoculum age and size. The subsequent experiments with the optimized conditions yielded 6.97 g/L of rhamnolipid, which is very consistent with the prediction made by the model used.

Key words: Biosurfactants, inoculum age, inoculum size, *Pseudomonas nitroreducens*, response surface design, rhamnolipid.

INTRODUCTION

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bounding such as oil/water or air water interfaces (Desai and Banat, 1997). Interest in microbial surfactants has been steadily increased in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential application in the environmental protection, crude oil recovery, health care, and food processing industries (Cameotra and Makkar, 2004; Rodrigues et al., 2006).

Rhamnolipids are secondary metabolites (Ron and Rosenberg, 2001) which are produced at late log phase or at the onset of stationary phase (Santa-Anna et al., 2002; Raza et al. 2006). Among other parameters, one of the major factors guiding the production of these metabolites is the inoculum development (Zhang et al., 2002). The attainment of optimum growth in production stage mainly depends on the age and density of the seed culture since they can affect the overall production yield and cost of the fermentation process (Sen and Swaminathan, 2004; Mnif et al., 2012a). Old bacterial cultures are found to need a long period of adaptation, often followed by a poorer growth than would have been expected if a young culture had been used, so the transfer time or age and

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Table 1. Actual factor levels corresponding to the coded factor for inoculum age and size.

Variable	Code	Actual value				
		–2	–1	0	+1	+2
Primary inoculums Age (h)	X_1	12	24	48	72	96
Size [% (v/v)]	X_2	1	5	9	13	17
Secondary inoculum Age (h)[a]	X_3	0	4	12	20	28
Size [% (v/v)]	X_4	0	5	10	15	20

[a]In developing secondary inoculum age of 0 h, only the primary inoculum (5%v/v) was used as seed culture for the production of rhamnolipid using sterile mineral salts medium (MSM).

volume are of importance. In order to avert this, two-stage inocula is encouraged as it always leads to the activation of microbial strains and hence, a higher mean rate of synthesis of a product (Milner et al., 1997; Mnif et al., 2012a). Some reports have been presented on the effects of inoculum age and size on biosurfactants production (Sen and Swaminathan 2004; Mnif et al., 2012a).

The use of statistical optimization strategy based on response surface methodology (RSM) has been used by various investigators (Abbasi et al. 2013; Mnif et al., 2012b; Najafi et al., 2010; Rodrigues et al., 2006; Roldan-Carrillo et al., 2011). With a view to simultaneously reduce the number of experiments and obtaining the mutual interactions between the variables required for achieving the optimal experimental conditions, a 2^4 full factorial central composite design (using response surface methodology) was employed for experimental design and analysis of results (Khuri and Cornell, 1987; Montgomery, 1991). There is paucity of information on effects of inoculum development on rhamnolipid production by *Pseudomonas nitroreducens*. Therefore, the aim of this study was to apply response surface methodology (RSM) in the optimization of inoculum build-up for enhanced rhamnolipid production by *P. nitroreducens* isolated from petroleum-contaminated soil.

MATERIALS AND METHODS

Microorganism and inoculum development

P. nitroreducens MILB-8054A with accession number (AY297786) used in this study was previously isolated from petroleum-contaminated soil in Awka, Nigeria (Onwosi and Odibo, 2012). The primary inoculum was prepared by transferring a loopful of bacteria from the slant to 250 mL Erlenmeyer flask containing 50 mL of mineral salts medium and 2% glucose as carbon source, and incubated in an orbital shaker. The secondary inoculum was developed by transferring a known volume of primary inoculum into mineral salts medium (MSM) and incubated. The size and age of the primary and secondary inocula transferred in each case were determined by the experimental design (Table 1).

Growth medium and fermentation conditions

A mineral salts medium (MSM), according to Pruthi and Cameotra (1997), with few modifications and containing the following components (g /L), was used for the growth of the isolate: KH_2PO_4, 2.0; K_2HPO_4, 5.0; $(NH_4)_2SO_4$, 3.0; $NaNO_3$, 2.0; NaCl, 0.10; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.01. It also contained trace elements solution having the following components (mg/L) $ZnSO_4.7H_2O$, 5.25; $MnSO_4.4H_2O$, 200; $CuSO_4.5H_2O$, 70.5; $NH_4MoO_4.2H_2O$; 15; $CoCl_2.6H_2O$, 200; H_3BO_3, 15. The pH of the medium was adjusted to 6.8 using 1 M NaOH before autoclaving at 121°C for 15 min. Glucose (2%) was used as the sole carbon source which was sterilized (110°C for 10 min) separately and aseptically added to the flasks containing MSM. Laboratory scale biosurfactant production was carried out in 250 mL Erlenmeyer flasks (containing 50 mL of medium inoculated with appropriate amounts of inoculum according to experimental design) incubated in an orbital shaker (180 rpm) at 30°C for 7 d.

Rhamnolipid quantification

The orcinol assay was used for direct estimation of the amount of glycolipids in the samples (Chandrasekaran and Bemiller, 1980). Extracellular glycolipids concentration was evaluated in triplicate by measuring the concentration of rhamnose: 0.33 mL of the culture supernatant was extracted twice with 1 mL diethyl ether. The ether fractions were evaporated to dryness and 0.5 mL of H_2O was added. To 1 mL of each sample 9 mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80°C the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg/mL).

Experimental design and optimization by response surface methodology for inoculum development.

A 2^4 full-factorial central composite design (Montgomery, 1991) for four test variables, each at five levels with eight star points and six replicates at the centre points employed to fit a quadratic model, indicated 30 experiments were required for the process (Sen and Swaminathan, 2004). The central values chosen for the experimental design were as follows: Primary inoculum age = 48 h, primary inoculum size = 9%, secondary inoculum age = 12 h, secondary inoculum size = 10%. In developing the regression equation, the test variables were coded according to the equation.

$$x_i = \frac{Xi - Xi^*}{\Delta Xi} \quad = 1,2\ldots\ldots K \qquad (1)$$

Where, Xi is the coded value of the ith independent variable, Xi is the uncoded value of the ith independent variable, Xi^* is the unco-

uncoded value of the *ith* independent variable at the centre point and ΔXi is the step change value. Table 1 shows the actual factor levels corresponding to coded factor levels. The second – order polynomial model was fitted to response giving an equation term:

$$Y_i = b_0 + b_1x_{1i} + b_2x_{2i} + b_3x_{3i} + b_4x_{4i} + b_{11}x^2_{1i} + b_{22}x^2_{2i} + b_{33}x^2_{3i} + b_{44}x^2_{4i} + b_{12}x_{1i}x_{2i} + b_{13}x_{1i}x_{3i} + b_{14}x_{1i}x_{4i} + b_{23}x_{2i}x_{3i} + b_{24}x_{2i}x_{4i} + b_{34}x_{3i}x_{4i} \quad (2)$$

Where, Y_i is the predicted response. The coefficient b_0 is the free term called intercept, x_i's are the input variables; 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 interactive terms.

Data analysis

The MATLAB software package was used for regression and graphical analysis of the experimental data obtained. The significance of the regression coefficient was tested by *t*- test. The level of significance was given as values Prob. > F. The optimal values of the starter cultures were obtained by solving the regression equation using a Quasi- Newton line search.

RESULTS AND DISCUSSION

Optimization of rhamnolipid production by *P. nitroreducens* using two-stage inocula

The statistical treatment combinations of the test variables along with the measured response values corresponding to each combination are shown in Table 2. The summary of the analysis of variance (ANOVA) representing the results of the quadratic response surface model fitting are shown in Table 3. ANOVA is an indispensible tool to ascertain the adequacy and significance of the model. The test of significance was checked at 5% level. The determination coefficient R^2 (a measure of goodness of fit of the model) was significant at the level of ~ 70%, implying that the model was unable to explain 30% of the total variations. High value of the correlation coefficient (S = 1.203) suggests a very good correlation between the experimental results and the predicted values. The normal probability plot of the "studentized" residuals is an important tool to detect and explain the systematic departures from the assumptions that errors are normally distributed and are independent of each other (Montgomery, 1991). In this study, the plot of "studentized" residuals for rhamnolipid production shows that the majority of the points are on the straight line which indicates that the assumption of normality was not violated (Figure 1).

The application of response surface methodology yielded the following regression equation (corresponding to Equation 1) which is an empirical relationship between the amount of rhamnolipid produced and the test variables in the coded units:

$$Y_i = 3.60 + 0.51X_1 + 0.52X_2 + 0.43X_3 + 0.23X_4 - 0.47X_1^2 - 0.02X_2^2 - 0.22X_3^2 - 0.25X_4^2 - 0.32X_1X_2 + 0.14X_1X_3 - 0.13X_1X_4 - 0.45X_2X_3 - 0.12X_2X_4 - 1.02X_3X_4 \quad (3)$$

Where, Y_i is the predicted rhamnolipid yield, X_1 = primary inoculum age, X_2= primary inoculum size , X_3= secondary inoculum age and X_4= secondary inoculum size.

The Students *t* -distribution and the corresponding *P*-values, along with the parameter estimate, are given in Table 4. The *P*-values were used as a tool to check the significance of each of the coefficients which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of *P*-value, the more significant is the corresponding coefficient (Khuri and Cornell, 1987; Du, 2003). The low probability values of the coefficient of primary inoculum age (P= 0.026 < 0.05) and size (P= 0.035< 0.05) make their first order main effects very significant. This implies that the production of rhamnolipid will be directly influenced by any change in the levels of the variables. The quadratic effects of the primary and secondary inocula age and size, respectively, are not significant (*P*- values are greater than 5% level of significance). However, the mutual interaction between secondary inoculum age and size (X_3X_4) is very strong, owing to the low probability value of the interactive terms (P= 0.004 < 0.05). Other mutual interactions (for example, primary inoculum age and secondary size (X_1X_4), and primary inoculum size and secondary inoculum size (X_2X_4)) are not significant. This suggests that secondary inoculum age and size should be well controlled to ensure a better performance during rhamnolipid production.

The three-dimensional response surface plots are generally the graphical representation of the regression equation (Gu et al., 2005). The response surface plots have been drawn with the vertical axis representing rhamnolipids production (Y) and two horizontal axes representing the coded levels of the two most significant factors for response (primary and secondary inocula age and size). Factors not represented by the two horizontal axes are fixed at their optimum levels (Figures 2 and 3).

The maximum values of the test variables were obtained by using Quasi Newton line search which is included in the MATLAB Regression software. The maximum point Y_i (Eq. 3) obtained for rhamnolipids production was (X_1, X_2, X_3, X_4) = (1.7908, -2, 2, -2). Recording the coded levels back to the original levels, the following results were obtained: Primary inoculum age = 61 h, primary inoculum size = 1%, secondary inoculum age = 28 h, secondary inoculum size = 20%. The maximum rhamnolipids yield that can be achieved, according to the model prediction under optimum experimental conditions, is 7.944 g/L. The experimental verification of the prediction of the model yielded 6.97 g/L of rhamnolipids. This confirms that the experimental result is consistent with the prediction. Mnif et al. (2012a) reported that adjusting the levels of the two-stage inocula strategy, lipopeptide production was effectively enhanced to almost 3.4 g/l as estimated gravimetrically when compared to the production yield described under non-optimized inocula conditions reported in

Table 2. Central composite design matrix of four factors in coded and natural units along with their responses for inoculum age and size.

Obs.	Coded value				Primary inoculum		Secondary inoculum		Y^a
Number	x_1	x_2	x_3	x_4	X_1, Age(h)	X_2,size[%(v/v)]	X_3, Age(h)	X_4,size[%(v/v)]	g/L)
1	-1	-1	-1	-1	24	5	4	5	0.21
2	1	-1	-1	-1	72	5	4	5	0.39
3	-1	1	-1	-1	24	13	4	5	0.72
4	1	1	-1	-1	72	13	4	5	3.72
5	-1	-1	1	-1	24	5	20	5	2.10
6	1	-1	1	-1	72	5	20	5	3.87
7	-1	1	1	-1	24	13	20	5	2.64
8	1	1	1	-1	72	13	20	5	3.81
9	-1	-1	-1	1	24	5	4	15	0.96
10	1	-1	-1	1	72	5	4	15	4.17
11	-1	1	-1	1	24	13	4	15	5.04
12	1	1	-1	1	72	13	4	15	2.91
13	-1	-1	1	1	24	5	20	15	0.42
14	1	-1	1	1	72	5	20	15	2.73
15	-1	1	1	1	24	13	20	15	1.08
16	1	1	1	1	72	13	20	15	1.95
17	-2	0	0	0	12	9	12	10	1.53
18	2	0	0	0	96	9	12	10	1.77
19	0	-2	0	0	48	1	12	10	2.25
20	0	2	0	0	48	17	12	10	4.23
21	0	0	-2	0	48	9	0	10	0.39
22	0	0	2	0	48	9	28	10	4.65
23	0	0	0	-2	48	9	12	0	1.71
24	0	0	0	2	48	9	12	20	3.15
25	0	0	0	0	48	9	12	10	3.18
26	0	0	0	0	48	9	12	10	3.21
27	0	0	0	0	48	9	12	10	3.18
28	0	0	0	9	48	9	12	10	3.15
29	0	0	0	0	48	9	12	10	3.21
30	0	0	0	0	48	9	12	10	3.15

[a]Rhamnolipids yield.

Table 3. Parameter estimate of the quadratic response surface model for rhamnolipid production.

Model term	Parameter estimate	Standard deviation	t-value	P-value
Constant	3.604	0.4909	7.341	0.000
X1	0.509	0.2455	2.072	0.026
X2	0.523	0.2455	2.130	0.035
X3	0.429	0.2455	1.749	0.101
X4	0.225	0.2455	0.918	0.373
X1*X1	- 0.466	0.2296	- 2.028	0.061
X2*X2	-0.015	0.2296	-0.066	0.948
X3*X3	-0.219	0.2296	-0.955	0.355
X4*X4	-0.245	0.2296	-1.066	0.303
X1*X2	-0.317	0.3006	-1.053	0.309
X1*X3	0.138	0.3006	0.459	0.653

Table 3. Contd.

X1*X4	-0.125	0.3006	-0.417	0.683
X2*X3	-0.453	0.3006	-1.506	0.153
X2*X4	-0.121	0.3006	-0.403	0.693
X3*X4	-1.018	0.3006	-3.386	0.004

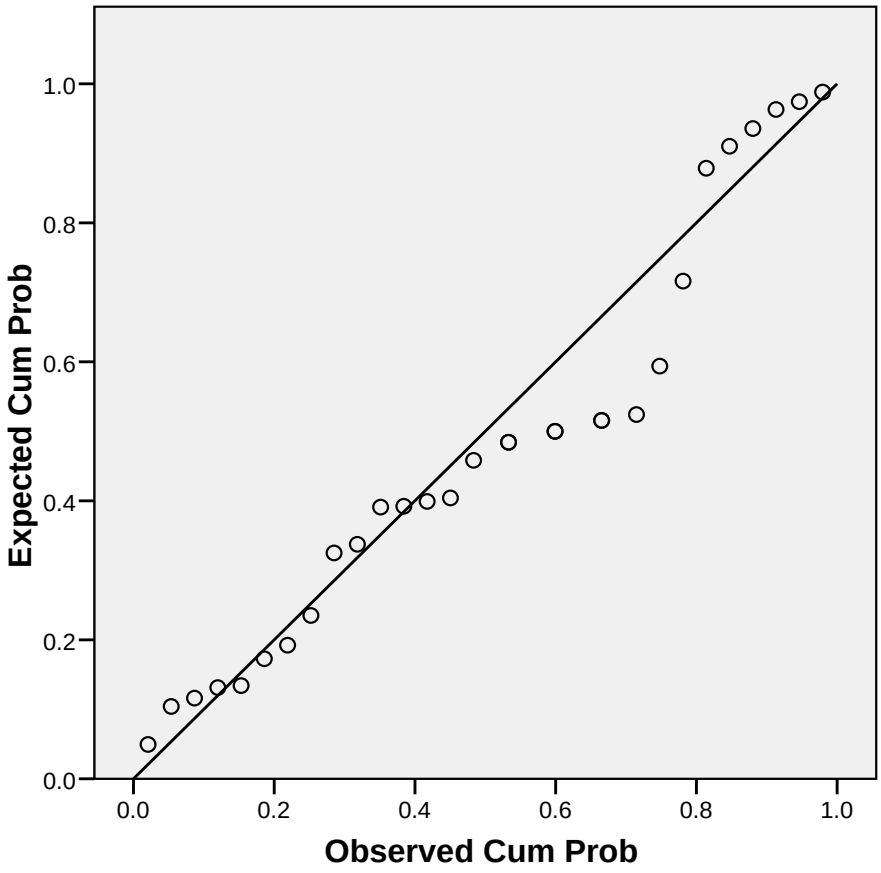

Figure 1. Normal probability plot of the residuals for rhamnolipid production showing that majority of the points lie on the straight line which indicates that the assumption of normality is not violated.

Table 4. Analysis of variance (ANOVA) table for the quadratic response surface model for rhamnolipids production.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	48.2023	48.2023	3.44302	2.38	0.053
Linear	4	18.4060	18.4060	4.60151	3.18	0.044
Square	4	7.5457	7.5457	1.88642	1.30	0.313
Interaction	6	22.2505	22.2505	3.70842	2.56	0.065
Residual error	15	21.6911	21.6911	1.44607		
Lack-of-fit	10	21.6865	21.6865	2.16865	2E+03	0.000
Pure error	5	0.0046	0.0046	0.00092		
Total	29	69.8933				

S = 1.203, R^2 = 0.70, adjusted R^2 = 0.44.

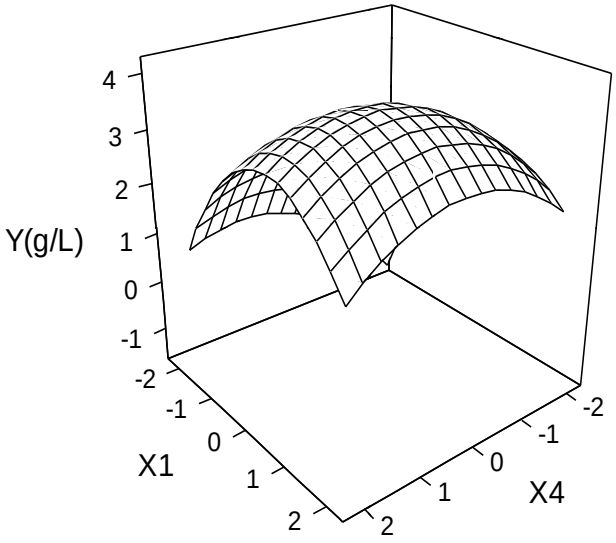

Figure 2. Response surface for the effect of primary inoculum age (X_1) and secondary inoculum size (X_4) on the rhamnolipid production.

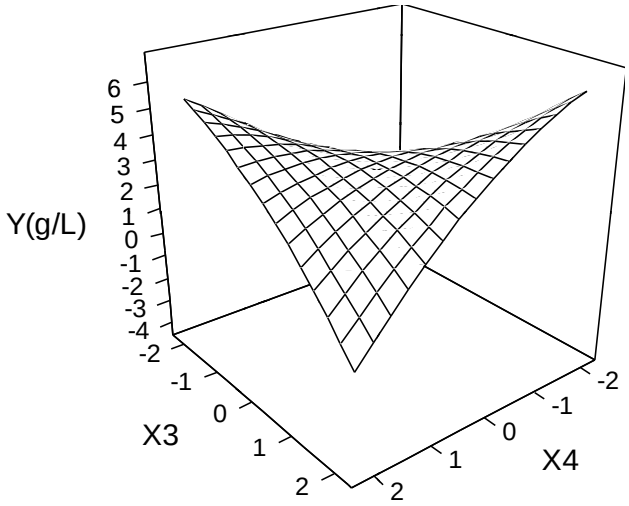

Figure 3. Response surface for the effect of secondary inoculum age (X_3) and size (X_4) on rhamnolipid production.

their previous work. Also, Xi et al. (2012) concluded that, the fermentation process efficiency generally increases as a function of initial stage < two-stage < multi-stage inoculations. This can be beneficial for fermentation process enhancing microorganisms growth and metabolite production (Sen and Swaminathan, 2004; Tafreshi et al. 2010).

Conclusion

The statistical modeling based on response surface modeling was used successfully in the present study to optimize two-stage inocula development towards improved rhamnolipid production by a newly isolated *P. nitroreducens* from petroleum-contaminated soil. The model predicted a maximum rhamnolipid biosurfactant yield of 7.944 g/L with optimal age and size of the primary inoculum at 61 h and 1% (v/v) and secondary inoculum 28 h and 20%, respectively owing to strong interaction between secondary age and size. This shows that a longer primary inoculum age with lower primary inoculum size and shorter secondary inoculum age with a shorter secondary inoculum size are necessary for optimized rhamnolipid production. Verification of the model prediction using experiments showed maximum rhamnolipid yield of 6.97 g/L. The pattern of rhamnolipid production was found to be growth-associated type and rhamnolipid surfactant reduced the surface tension of water from 72 to 37mN/m.

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