Annals of Microbiology, **57 (4)** 571-575 (2007)

Optimisation of rhamnolipids produced by *Pseudomonas aeruginosa* 181 using Response Surface Modeling

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Received 9 May 2007 / Accepted 27 September 2007

Abstract - This work investigated the optimisation of the fermented culture medium for maximisation of rhamnolipids production produced by *Pseudomonas aeruginosa* 181 using Response Surface Modeling (RSM). A two full factorial central composite experimental design was used in the design of experiments and in the analysis of results. This procedure limited the number of actual experiments performed while allowing for possible interactions between the parameters (pH, stirring rate, casamino acid concentration and incubation period) on the production of biosurfactants. Production carried out at larger volumes of one litre using Bioreactor under RSM-optimised conditions yielded 3.61 g l^{-1} of products after purification by acid precipitation.

Key words: biosurfactant, rhamnolipid, *Pseudomonas aeruginosa,* production, RSM.

INTRODUCTION

Microbial-derived surfactants or biosurfactants are produced by a wide variety of microbes and are amphipathic molecules with a hydrophilic and a hydrophobic domain. These traits of biosurfactants can accumulate at interfaces to form micelles that lower the surface tension and thereby enhance the solubility of poorly soluble compounds in water (Kuiper *et al*., 2004). Biosurfactants are best known for enhancing the bioavailability of hydrophobic soil pollutants such as polycyclic aromatic hydrocarbons (PAHs). By reducing the surface tension between water and hydrophobic surfaces the formation of emulsions of hydrophobic xenobiotics in water is enhanced, thereby increasing the growth surfaces for bacteria (Garcia-Junco *et al*., 2001). Although biosurfactants have in common their amphipathic character, their primary structures show a wide diversity. An intensely studied group of biosurfactants is that of the glycolipids, with rhamnolipids produced by *Pseudomonas* bacteria as a major representative (Stanghellini and Miller, 1997). Rhamnolipids were first isolated from *Pseudomonas aeruginosa* and described by Jarvis and Johnson (1949). These compounds are predominantly constructed from the union of one or two rhamnose sugar molecules and one or two β-hydroxy (3-hydroxy) fatty acids. Rhamnolipids with one sugar molecule are referred to as mono-rhamnolipids, while those with two sugar molecules are di-rhamnolipids (Gunther *et al*., 2005). Response surface modeling (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 optimisation of a factor for desirable responses. The aim of this work is the optimisation of the culture condition for rhamnolipid production by *Pseudomonas aeruginosa* 181.

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MATERIAL AND METHODS

Microorganism. *Pseudomonas aeruginosa* 181 was isolated from contaminated soils of a motor workshop in Serdang, Selangor, Malaysia (Ali, 1998). The strain was grown at 37 °C for 18 h and maintained at 4 °C on TSA plates and alternatively at -80 °C.

Experimental design. A four-variable fractional factorial (2^{4-1}) design was employed. The variables and their respective levels were selected based on the preceding one-variable-at-a-time study. Both their actual and corresponding coded values are presented in Table 1.

Table 2 represents the design matrix of the actual experiments carried out for developing the model. The matrix is made up of a 2^{4-1} fractional factorial design (Runs 1, 5, 9, 10, 12, 15, 17, 19) combined with 5 centre points for error estimation (Runs 4, 14, 18, 20, 21) and 8 axial points where one variable is set at an extreme level (±

1.68) while keeping the others at their centre point values (Runs 2, 3, 6, 7, 8, 11, 13, 16). Triplicate experiments were set up for each run with all 21 runs performed in random order. The experiments were prepared by various pH 6.75 to 7.25. Concentration of the casamino acid was varied between (0 to 15.75 g l^{-1}) while the stirring rates were varied from (0 to 318 rpm) and the incubation period was varied from (0 to 5 day). The required temperature was kept at 37 °C) with 5% (v/v) size of inoculation while the response obtained was the yield of the production.

Statistical and graphical analyses. The regression analyses and statistical significance of the models were tested using (ANOVA) and response surfaces were generated using the software Design Expert Version 6.0.4 (Stat-Ease Inc., Statistics Made Easy, Minneapolis, MN, USA).

Optimisation of production and model validation. For every model selected, a set of optimal reaction conditions was generated using the numerical optimisation function in the Design Expert software based on an objective function called desirability (Design Expert Version 6.0.4 User's Guide). The overall desirability (D) is the geometric (multiplicative) mean of all individual desirability (d that range from 0 (least) to 1 (most). If any of the responses fell outside their desirability range, the overall function becomes zero. Experiments were then carried out under the recommended conditions and the resulting yields compared to those predicted by the software. Optimised product was scaled-up volumetrically, run in one litre Bioreactor, and the products isolated and purified.

Analytical methods.

Recovery of biosurfactant. Recovery of biosurfactant was done as described by Miller (Personal communication) with modification. Cells were removed from the culture fluid by centrifugation at 15300 *x g* for 15 minutes. Crude biosurfactant from the supernatant was then isolated by

TABLE 1 - Coded and actual levels of variables for design of experiment

Variables		Units	Coded level of variable				
			-1.682	-1	0	1	1.682
			Actual levels of variables				
X_1	pH		6.75	6.85	7	7.15	7.25
X_2	Stirring rates	rpm	0	50	150	250	318.8
X_3	Casamino acid Concentration	g l^{-1}	0	1	6.5	12	15.75
X_4	Incubation	day	0	1	3	5	6.36

TABLE 2 - Coded and actual level combinations for a four-variable fractional factorial design

Run	Actual Levels				Coded Levels			
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4
1	7.15	250	2	1	1	1	1	-1
2	7.15	250	1	1	1	1	-1	-1
3	7.15	50	12	5	1	-1	1	1
4	6.85	250	1	5	-1	1	-1	1
5	7.15	50	1	5	1	-1	-1	1
6	6.85	50	12	1	-1	-1	1	-1
7	6.85	250	12	5	-1	1	1	1
8	6.85	50	1	1	-1	-1	-1	-1
9	6.75	150	6.5	3	-1.68	0	0	0
10	7.25	150	6.5	3	1.68	0	0	0
11	7	0	6.5	3	0	-1.68	0	0
12	7	318	6.5	3	0	1.68	0	0
13	7	150	0	3	0	0	-1.68	0
14	7	150	15.75	3	0	0	1.68	0
15	7	150	6.5	0	0	0	0	-1.68
16	7	150	6.5	6.36	0	0	0	1.68
17	7	150	6.5	3	0	0	0	0
18	7	150	6.5	3	0	0	0	0
19	7	150	6.5	3	0	0	0	0
20	7	150	6.5	3	0	0	0	0
21	7	150	6.5	3	0	0	0	0

X_1: pH, X_2: stirring rate, X_3: casamino acid concentration, X_4: incubation period.

adding concentrated hydrochloric acid to the supernatant until the pH reached to < 2.0. The resulting flocculate was separated from the solution by centrifuging at 15300 *x g* for 15 minutes. Then pellet was obtained and dissolved in generous 10:1 chloroform to methanol, followed by removing the organic phase and recovering the rhamnolipids by removing the solvent by rotoevaporation. The remaining aqueous phase was extracted twice using the same chloroform/methanol mixture.

Quantification of biosurfactants. Biosurfactants were quantified by expressing rhamnolipids in term of mg l^{-1} rhamnose. Rhamnose was estimated from the residue dissolved in $NaHCO_3$ by the cysteine hydrochloride reaction (Patel and Desai, 1997).

RESULTS AND DISCUSSION

Figure 1, show the response surface plots as functions of pH versus stirring rate, casamino acid concentration, and incubation period, respectively, for rhamnolipid production. Several trends were observed for the predicted combined effects of pH and stirring rate (Fig. 1a) at a fixed casamino acid concentration of 6.5 g l^{-1} and an incubation period of 3 day. The negative effects of pH medium predominated over the positive effects of stirring rate with the use of acidic medium. When carrying out the medium at alkaline pH, the positive effect of stirring rate was clearly evident. The pH was seen to play a more important role in that irrespective stirring rate. Increasing pH was paralleled with large increments in yield. This is consistent with enhanced

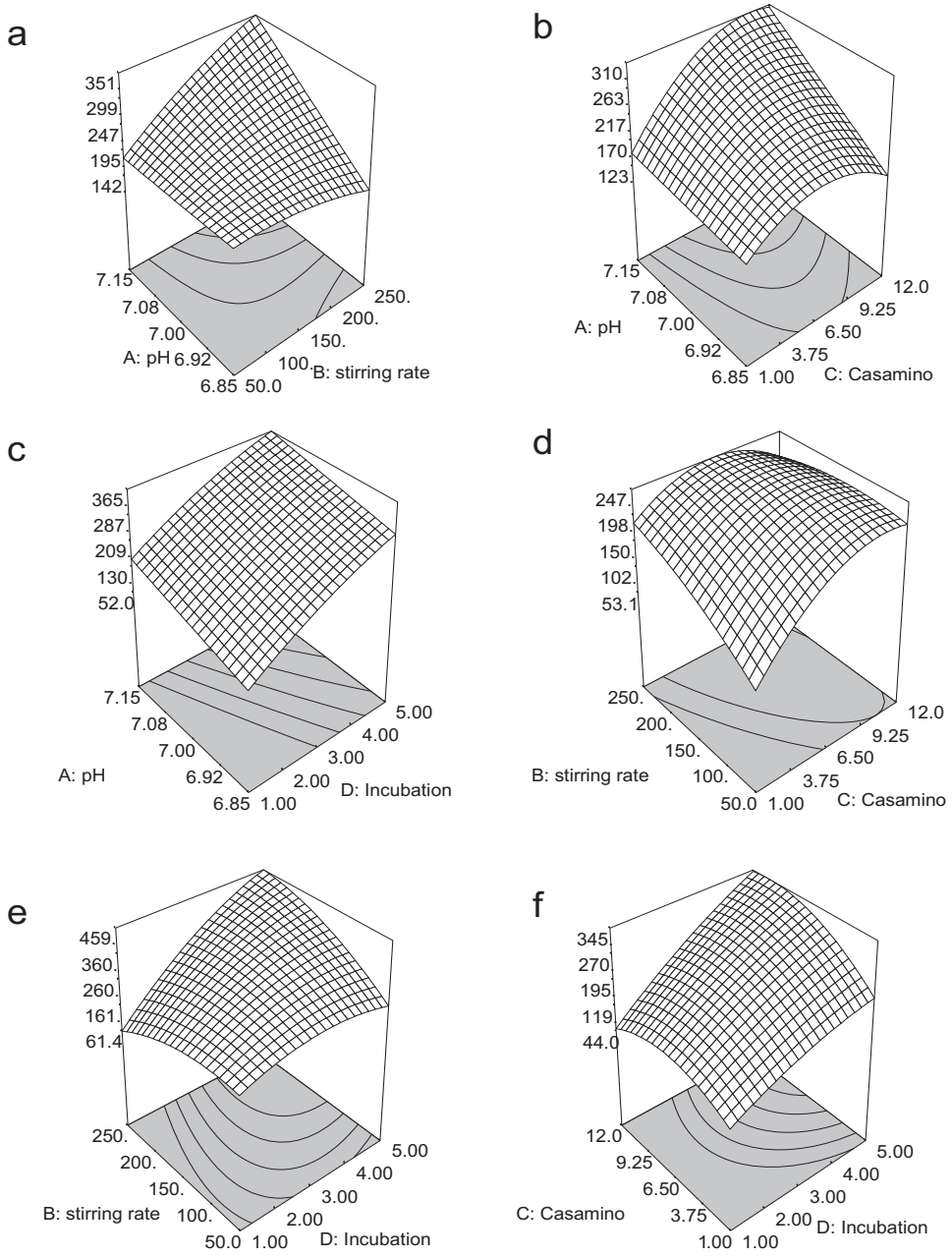

FIG. 1 - Response Surface Plots for Rhamnolipid. a: pH versus stirring rate (AB), b: pH versus casamino acid concentration (AC), c: pH versus incubation period (AD), d: stirring rate versus casamino acid concentration (BC), e: stirring rate versus incubation period (BD), f: casamino acid concentration versus incubation period (CD).

TABLE 3 - Optimal conditions derived by RSM for rhamnolipid production by *Pseudomonas aeruginosa* 181

Optimal condition				Predicted yield ($g\ l^{-1}$)*	Actual yield ($g\ l^{-1}$)**	Relative deviation (%)***
X_1	X_2 (rpm)	X_3 ($g\ l^{-1}$)	X_4 (day)			
7.15	250	5.33	2.69	3.66	3.61	1.36

X_1: pH, X_2: stirring rate, X_3: casamino acid concentration, X_4: incubation period.
* Predicted based on respective models developed; ** average value of triplicate runs; *** refer to this equation: Relative deviation % = (Predicted Yield - Actual Yield/Predicted Yield) x 100

substrate utilised by the bacteria and in more acidic media as well as at the alkaline media; the best yields were achieved at the highest pH (7.15) in the high rate of stirring (250 rpm). Interaction of pH and casamino acid concentration is shown in Fig. 1b. Patel and Desai (1997) reported that *Pseudomonas aeruginosa* utilised molasses at pH range between 7.00-7.20. The positive effect of pH with interaction of incubation period was detected by high yields of rhamnolipids (Fig. 1c). The combined dual actions of stirring rate versus casamino acid concentration on rhamnolipids production are illustrated in Fig. 1d. Irrespective of the stirring rate, yields were expected to increase linearly and significantly with incubation period (Fig. 1e). In contrast, increasing stirring rate at all levels of the incubation period led to less significant improvements in yield. The lower yields predicted in short period of incubation reflect the action of bacterium utilising substrate irrespective of the rate of stirring rate, higher yields were expected which also seem to respond positively with increasing incubation period. Predicted response surfaces for interactions of casamino acid concentration and incubation period for rhamnolipids production are presented in Fig. 1f. The low levels of biosurfactants produced have greatly hampered research on the role of biosurfactants; however, a number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. Recent developments in the area of optimisation of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive. These developments include the use of a fed batch technique in which the yield of rhamnolipid production by *Pseudomonas aeruginosa*. Haba *et al.* (2000) observed 2.7 g rhamnolipid l^{-1} from waste frying oil by *P. aeruginosa* 47T2 NCIB 40044. Rahman *et al.* (2002) observed 4.31 g rhamnolipid l^{-1} by *P. aeruginosa* DS10-129 at 288 h of incubation. In our study, the product yield 3.61 g rhamnolipids l^{-1} higher than 0.472 g rhamnolipids by *P. aeruginosa* EBN-8 (Raza *et al.,* 2007), 0.382 g rhamnolipid by *P. aeruginosa* 44T1 (Robert *et al.,* 1989), 0.058 g rhamnolipid by *Pseudomonas* sp. JAMM (Mercade *et al.,* 1993), 0.405 g rhamnolipid by *P. aeruginosa* UW-1 (Sim *et al.,* 1997), 0.089 g rhamnolipid by *P. aeruginosa* BS2 (Babu *et al.,* 1996) and 1.5 g by *Pseudomonas aeruginosa* AT10 (Abalos *et al*., 2002) which reported that all the test variables (carbon source, nitrogen source and phosphate) have a strong effect on rhamnolipid production. The yield of sophorolipids by *T. bombicola* increased from 0.37 g per gram substrate in batch culture to 0.6 g per gram substrate (Rodrigues *et al.,* 2006). In the present study an increase about five times in production of biosurfactant per gram cell dry weight.

The optimal conditions for the rhamnolipid production by *Pseudomonas aeruginosa* 181 were predicted using the optimisation function of the Design Expert software (Table 3). The relative deviation values obtained (1.36%) are comparable to those reported in RSM studies on enzymatic synthesis 0.7% (Hamsaveni *et al*., 2001). These models can therefore be used to predict rhamnolipid yields under any given conditions within the experimental range.

Acknowledgement
The Ministry of Science Technology and Environmental Malaysia (IRPA No: 09 02 04 0184) and Kulliyyah of Nursing, International Islamic University Malaysia.

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