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Research Article

Optimization of Operational Parameters in Rhamnolipid Production by *Pseudomonas aeruginosa* MM1011 in a Miniaturized Shaken Bioreactor

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Abstract

A rhamnolipid–type biosurfactant production by *Pseudomonas aeruginosa* MM1011 was studied in a miniaturized shaken bioreactor. The operational parameters, affecting the biosurfactant production in shaken bioreactors, such as shaking frequency (200, 250, 300 rpm), filling volume (50, 75, 100 ml), and aeration rate (0.2, 0.6, 1 vvm), were optimized using response surface methodology. The optimization process conducted based on three different response variables (surface tension, rhamnolipid concentration, and emulsification activity). The best results were achieved at agitation rate of 292 rpm, filling volume of 50 ml, and aeration rate of 1 vvm. Also, the results indicated that all of the three factors were effective parameters in biosurfactant production and the surface tension, rhamnolipid production and emulsification activity under optimum conditions, were measured 31.00 mN/m, 1.89 g/L and 80.23 %, respectively.

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Introduction

Microbial surface active agents or biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic moieties. Due to unique molecular structure, they occur preferentially at the interfaces between an organic phase and an aqueous phase and find various applications involving the reduction of surface and interfacial tensions [1-3]. In recent years, attention has been directed towards biosurfactants owing to their several advantages over their chemical counterparts, such as structural diversity, biodegradability, low toxicity, high foaming, high selectivity, better environmental compatibility, efficient functionality at extreme conditions of temperature, pH, and salinity, and also ability to be synthesized from renewable feed-stocks [4-6].

A large variety of bacteria are able to produce different kinds of biosurfactants. *Pseudomonas aeruginosa* is one of the most commonly isolated bacterium which is well known for its great ability to produce rhamnolipid biosurfactant [7], and therefore, is a promising candidate for the large scale production of this type of biosurfactant [8]. Rhamnolipids which are glycolipid-type biosurfactants [9], are among the most effective surfactants that have significant tensioactive and emulsifying properties [10, 11] with immense potential in microbial enhanced oil recovery (MEOR), pharmaceutical industry, and particularly inbioremediation [12-14]. One of the various kinds of bioreactors that can be used to produce biosurfactants is shken bioreactor.

Shaken bioreactors are widely applied in basic bioprocess development and optimization projects which require a high number of experiments to be conducted in parallel [15, 16]. According to advantages such as ease of handling [17], simple mechanical design [18], low investment and operational costs [19], and needing no supervision[20], these bioreactors have proven to be invaluable and at the same time irreplaceable tools in bioindustries [21].

There are a number of operational parameters controlling biosurfactant production, which are required to be maintained within a certain range in order to achieve maximum productivity. In this regard, agitation rate, filling volume, and aeration rate are of prime importance in a shaking bioreactor for optimization of biosurfactant production. Due to the complex nature of biological processes, it is very difficult to predict distinctively the effects of all parameters, which may have multiple interactions. One of the best methodologies used to design the optimization experiments and analyze the experimental data is response surface methodology (RSM). RSM is an empirical technique employed for multiple regression analysis of quantitative data and solving multivariate equations simultaneously, which results in evaluating the effects of factors, building models, and searching for optimum conditions [22].

Central Composite Design (CCD) and Box-Behnken Design (BBD) are fractional factorial designs of RSM for

optimization of variables with a limited number of experiments [23].

Optimization of biosurfactant biosynthesis according to effective growth factors has been studied extensively, but there is dirt of information about optimal operational conditions for biosurfactant and particularly rhamnolipid production. Therefore, this work is primarily aimed at studying production of a rhamnolipid-type biosurfactant in a shaken bioreactor, in order to evaluate the relationships existing between the biosurfactant production and the operational parameters of agitation rate, filling volume and aeration rate and then statistically optimize these process variables to enhance the productivity by use of response surface methodology.

Materials and Methods

Microorganism and cultivation conditions

A strain of *P. aeruginosa* MM1011 used for biosurfactant production was provided from Persian Type Culture Collection (PTCC) of Iranian Research Organization for Science & Technology (IROST). The strain was confirmed by PTCC Identification report No. 1011 as *P. aeruginosa*, therefore it has been designated as *P. aeruginosa* MM1011.The microorganism was maintained at 4°C on nutrient agar plates throughout the experiments and subcultured every two weeks. For preparing pre-culture, a loopful of cells from a 17-h actively growing culture on a nutrient agar plate was transferred into a flask containing 50 ml nutrient broth and then it was incubated at 37°C in a shaking incubator (X-Climo-shaker ISF1-X, Kuhner, Switzerland) at 250 rpm for 15-18 h.

Fermentation experiments

For liquid fermentation, a 2% cell suspension with OD (optical density) of 1 at 600 nm, was inoculated in 500-ml flasks containing mineral salts medium. The mineral salts medium,containing (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17, was made [24] and amended with 6% glucose as a sole carbon substrate. The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool. After preparing the medium, various fermentation experiments carried out to exploit the effects of different operational conditions of agitation rates, aeration rate, and filling volumes on biosurfactant production. All fermentations were accomplished with incubation in 500 ml cylindrical flasks at 37°C for 7 days.

Experimental equipment

Aerobic microorganisms will encounter oxygen limitation if the oxygen transfer rate is smaller than the oxygen uptake rate in shaken bioreactors equipped with sterile closures [16, 25]. To avoid oxygen limitation in the shake flasks, it is essential to have a good understanding and estimation of the gas transfer conditions. The gas transfer coefficient of the sterile closure plays an important role in aeration, for the reduction of oxygen supply and the accumulation of CO_2 in the gas phase of the shaking flasks headspace. The value of the gas transfer coefficient of the sterile closure depends on the effective diffusion coefficient of oxygen and dimensions (length or/anddiameter) of the neck of the flasks [21]. In this study, the experiments were carried out using a kind of miniaturized bioreactors, so called ventilation flasks. Ventilation flasks which have been modeled [26], have different types, each type with a particular dimension of the diameter and height of the flask neck (Fig. 1). The necks of these flasks were filled with cotton wool with a constant density of 0.15 g/cm^3 that ensures the same value of effective diffusion coefficient of oxygen for the sterile closures in the ventilation flasks [16, 25]. In the present study 500 mlcylindrical flasks, with three types of closures, which ensure having three definite aeration rates for flasks, were applied. Moreover, the cylindrical shapes of the flasks have the advantage of geometrical resembling to industrial scale bioreactors and therefore can be helpful in scale up process. In Table 1 dimensions of the closures and their related aeration rates have been shown.

Analytical methods and measurements:

Determination of cell dry weight (Biomass) concentration

For the determination of biomass concentration, empty 15 ml falcon tubes were dried in an oven at 100°C for 48 h, placed in the desiccators for 0.5 h and then weighted by a precision balance. For each sample, a weighted Falcon Tube was filled with 10ml of culture medium and centrifuged for 20 min at 4°C and 5000 ×g (UNIVERSAL 320, Hettich, Germany) [27]. The supernatant was decanted and used for the analysis of the medium components. The humid pellet in the bottom of the Falcon Tube was dried for 24 h in the oven at 105°C and, afterwards, placed in the desiccators for 0.5 h and weighted for the determination of the cell dry weight.

Evaluation of surface activity

The surface tension of the free cell supernatant was determined using a tensiometer (Sigma 703, KSV, Finland), according to the Du Nouy ring method. The surface tension measurement was carried out at room temperature after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. For the calibration of the instrument, the surface tension of pure water was first measured. To increase the accuracy, the measurement was repeated at least three times, and an average of triplicates was used to express the surfacetension of the sample [28].

Determination of emulsification index (E_{24})

Emulsification activity was determined according to the method described [29]. Briefly, 2 ml of respective hydrocarbon was mixed with equal volume of culture supernatant and vigorously stirred for 5 min followed by incubation at 25°C for 24 h. The emulsification index (E_{24}) was determined as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm).

Rhamnolipid extraction

The extraction of rhamnolipid followed the method suggested [14, 30]. The collected culture supernatant was first centrifuged at 7000×g for 15 min to remove

P. aeruginosa cells. Rhamnolipid was then precipitated by acidification to pH 2.0. After centrifuged at 12,100×g for 20 min, the precipitate was extracted with chloroform–ethanol (2:1).

Figure 1. Ventilation flasks (right to left: No 1 to No 3).

Extract was then transferred to a round bottom flask connected to a rotary evaporator (RV 10 digital, IKA, China). The evaporation process was allowed to proceed until the precipitation turned to a honey-color, viscous consistency, which was then freezing dried (Alpha 1-2 LD Plus, CHRIST, Germany).

Table 1. Ventilation flasks specifications.

Flask type	Height (cm)	Diameter (cm)	Aeration rate (vvm)
No 1	2.12	2.8	1
No 2	5.2	2	0.6
No 3	7.5	1.5	0.2

Rhamnolipid quantification

Rhamnose concentration was measured in the cell free culture broth by the orcinol method described [31]. To this end, a volume of 333 µl of the acidified culture supernatant with HCl 1N was extracted twice with 1ml of diethyl ether. The fractions were pooled and evaporated to dryness (RV 10 digital, IKA) and then 0.5 ml of H_2O was added. To 100 µl of each sample 900 µl of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added; after heating for 30min at 80°C, the samples were cooled for 15min at room temperature and the absorbance was measured at 421 nm. The concentrations of rhamnose were calculated by comparing the data with those of rhamnose standards between 0 and 50 µg/ml. Rhamnolipid values were determined by multiplying rhamnose values by a coefficient of 3.4, obtained from the correlation y=[(0.0139x-0.0058)×0.68] of pure rhamnolipid/rhamnose [32].

Quantification of the glucose consumption

To determine the amount of glucose consumed by the bacterial culture, a Glucose (HK) Assay Kit (GAHK-20, Sigma Aldrich, USA) was applied. Sample preparation and glucose measurement followed the technical bulletin from the supplier, which was described [33].

Experimental design

While performing experiments, in order to decrease the error to a manageable percent and draw objective conclusions, it is essential to acquire the right data. A very efficient way to enhance the value and quality of research and reduce the time and cost of process development is through statistically designed experiments [34].

A designed experiment is usually a planned series of tests in which purposeful changes are made to the input variables of a process in order to observe and identify the reasons of changes in the output response [35]. In fermentation processes such as biosurfactant production, where operating variables interact and influence each other's effects on the response, the optimization method should account for these interactions in determining a set of optimal experimental conditions. In this study, with the aid of statistical software package, Design-Expert 7.0 (Stat-Ease, Inc., Minneapolis, MN, USA), 20 batch experiments (including five replicate measurements) were designed based on central composite design (CCD) algorithm (Table 2), using response surface methodology (RSM), which can well determine the mutual interactions between the test variables and their subsequent effect on the response.

Table 2. Results of Central Composite Design (CCD) for optimization of Rhamnolipid production.

Run	X_1: Filling Volume(ml)	X_2: Agitation Rate (rpm)	X_3: Aeration Rate (vvm)	R_1: Surface Tension (mN/m)	R_2: Emulsification Index (%)	R_3: Rhamnolipid Concentration(g/L)
1	50	200	0.2	41.26	66.81	1.22
2	100	200	0.2	49.33	57.34	0.83
3	50	300	0.2	32.70	76.34	1.67
4	100	300	0.2	45.37	63.43	1.07
5	50	200	1	39.54	68.46	1.31
6	100	200	1	47.40	59.28	0.92
7	50	300	1	28.60	80.31	1.89
8	100	300	1	43.48	65.70	1.20
9	50	250	0.6	30.12	79.14	1.77
10	100	250	0.6	44.92	63.71	1.10
11	80	200	0.6	46.38	61.49	0.98
12	80	300	0.6	42.18	67.18	1.23
13	80	250	0.2	43.54	65.26	1.16
14	80	250	1	41.02	68.52	1.30
15	80	250	0.6	42.61	66.73	1.21
16	80	250	0.6	42.60	66.75	1.19
17	80	250	0.6	42.62	66.73	1.20
18	80	250	0.6	42.59	66.72	1.23
19	80	250	0.6	42.61	66.71	1.21
20	80	250	0.6	42.63	66.74	1.22

Table 3 represents the design matrix of the variables in both coded and natural values indicating the range of the

independent parameters which was estimated according to the data from the preliminary conducted tests. As can be seen in the table, three variables (agitation rate, filling volume, and aeration rate), each at three levels of high (+1), middle (0), and low (−1) were investigated in order to optimize the rhamnolipid production.

Table 3. Experimental range and levels of the independent process variables.

Independent variable	Coded levels and the corresponding values		
	-1	0	1
***X1*: Filling volume (ml)**	50	75	100
***X2*: Agitation rate (rpm)**	200	250	300
***X3*: Aeration rate (vvm)**	0.2	0.6	1

In every designed experiment three different response variables (surface tension, emulsification index, and rhamnolipid concentration), were measured to increase the validity of optimization results (Table 3). Subsequently, data from CCD were subjected to a second-order multiple regression analysis to explain the behavior of the system using the least squares regression methodology and acquire the parameter estimators of the mathematical model, which can be expressed as follow:

$$Y = \beta_0 + \sum \beta_i \times X_i + \sum \beta_{ii} \times X_i^2 + \sum \beta_{ij} \times X_{ij} \quad (1)$$

Where *Y* is the response, *Xi* is the independent variable, β_0 is a constant, β_i is the slope or linear effect of the input factor, β_{ii} is the quadratic effect of input factor, and β_{ij} is the linear by linear interaction effect between the input factors.

After identification of promising conditions for rhamnolipid cultivation, under optimized conditions, different process parameters such as growth, surface tension, biosurfactant concentration, glucose consumption and emulsification activity were evaluated, at regular intervals, during 168 h, in order to assess the process of rhamnolipid production under optimum conditions.

Results and Discussion

Regression analysis

Regression analysis of the experimental data, and also plotting the response surface graphs were done by use of Design Expert software. Quadratic polynomial models, expressed in coded variables, were established based on regression coefficients to identify the relative sensitivity of the selected responses to the variables, as 2, 3 and 4 formulas.

where, *Y1*, *Y2* and, *Y3* represents the response values (i.e., surface tension, Emulsification Index, and rhamnolipid yield) respectively and *X1*, *X2*, and *X3* indicates the coded values pertaining to test variables (i.e., filling volume, agitation rate, and aeration rate), respectively. A positive sign of the coefficient represents a synergistic effect, while a negative sign indicates an antagonistic effect. As can be seen in the correlations, the relationship of each term with surface tension is opposite of the relationship of the same term with rhamnolipid yield and emulsification index; because it is desired to reach the as low as possible value for surface tension, while the best results for rhamnolipid yield and emulsification index are the highest ones.

$$(2)\quad Y_1 = 42.304 + 5.828(X_1) - 3.158(X_2) - 1.216(X_3) + 1.452(X_1)(X_2) + 0.250(X_1)(X_3) - 0.292(X_2)(X_3) - 4.326(X_1)^2 + 2.433(X_2)^2 + 0.433(X_3)^2$$

$$(3)\quad Y_2 = 67.018 - 6.160(X_1) + 3.958(X_2) + 1.309(X_3) - 1.108(X_1)(X_2) - 0.176(X_1)(X_3) + 0.331(X_2)(X_3) + 3.974(X_1)^2 - 3.115(X_2)^2 - 0.560(X_3)^2$$

$$(4)\quad Y_3 = 1.222 - 0.274(X_1) + 0.180(X_2) + 0.067(X_3) - 0.063(X_1)(X_2) - 0.011(X_1)(X_3) + 0.021(X_2)(X_3) + 0.193(X_1)^2 - 0.136(X_2)^2 - 0.011(X_3)^2 (4)$$

High values of the coefficients of *X1*, *X2*together with their quadratic terms indicate the significance of these variables on all the three responses, in spite of quadratic term of *X3* and interactive terms of *X1* and *X3* and that *of X2* and *X3*, which have low coefficients and consequently low significance.

Table 4 (A-C) shows the results of the quadratic response-surface model fitting, in the form of analysis of variance (ANOVA). ANOVA is required to test the significance and adequacy of the model and also to find out which factors had the most affecting interactions and which ones were most effective for achieving the best response. The presented P values were used as a tool to check the significance of every coefficient which, in turn, is necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient.

Generally, P-values less than 0.050 indicate significant terms and values greater than 0.10 specify insignificant terms. Thus, in this case, variables *X1*, *X2*, *X3*, *X1X2*, *X12*, and *X22*, all are significant terms while *X32*, *X1X3*, and *X2X3* are insignificant in the three models and can be omitted from the models to improve them.

Regression analysis also revealed the coefficients of determination (R^2) and adjusted coefficients of determination ($AdjR^2$) for the presented models. The closer the value of $R^2$is to 1, the better is the correlation between the observed and the predicted values. The values of coefficients imply that almost 97% of the variability in the responses could be explained by the related second-order polynomial prediction equations (Eq. 1-3).

Figure 2 (A-C) depict the predicted vs. experimental plots for the results of surface tension, emulsification index, and the rhamnolipid yield.

Table 4. ANOVA of the Model for (A): Surface Tension, (B): Emulsification Index, and (C): Rhamnolipid Yield.

(A)

Term	Degree of Freedom	Mean Square	F-value	P-value
Model	9	58.70	50.43	<0.0001
X_1	1	339.66	291.84	<0.0001
X_2	1	99.73	85.69	<0.0001
X_3	1	14.79	12.70	0.0051
X_1X_2	1	16.88	14.50	0.0034
X_1X_3	1	0.50	0.43	0.5270
X_2X_3	1	0.68	0.59	0.4609
X_1^2	1	51.47	44.23	<0.0001
X_2^2	1	16.29	13.99	0.0038
X_3^2	1	0.52	0.44	0.5201
Residual	10	1.16	-	-

R^2= 0.9784 R^2(adjusted)= 0.9590

(B)

Term	Degree of Freedom	Mean Square	F-value	P-value
Model	9	68.46	58.97	<0.0001
X_1	1	379.46	326.88	<0.0001
X_2	1	156.66	134.95	<0.0001
X_3	1	17.13	14.76	0.0033
X_1X_2	1	9.83	8.47	0.0155
X_1X_3	1	0.25	0.21	0.6535
X_2X_3	1	0.88	0.76	0.4049
X_1^2	1	43.44	37.42	0.0001
X_2^2	1	26.69	22.99	0.0007
X_3^2	1	0.86	0.74	0.4086
Residual	10	1.16	-	-

R^2= 0.9815 R^2(adjusted)= 0.9649

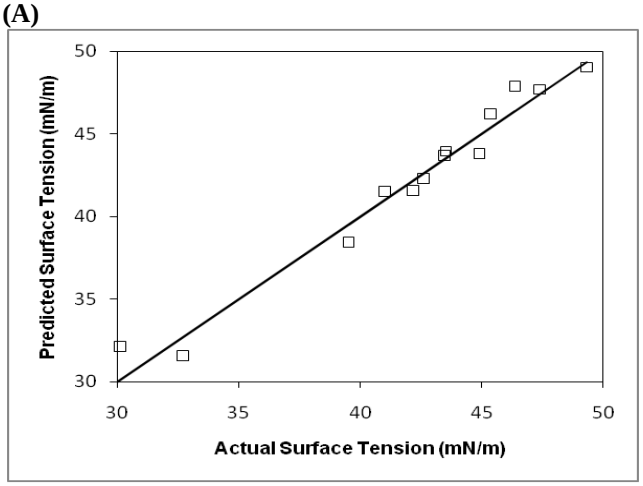
(C)

Term	Degree of Freedom	Mean Square	F-value	P-value
Model	9	0.14	64.00	<0.0001
X_1	1	0.75	339.00	<0.0001
X_2	1	0.32	146.30	<0.0001
X_3	1	0.045	20.27	0.0011
X_1X_2	1	0.033	14.68	0.0033
X_1X_3	1	1.013E-003	0.46	0.5143
X_2X_3	1	3.613E-003	1.63	0.2304
X_1^2	1	0.10	46.56	<0.0001
X_2^2	1	0.051	23.09	0.0007
X_3^2	1	3.551E-004	0.16	0.6973
Residual	10	2.215E-003	-	-

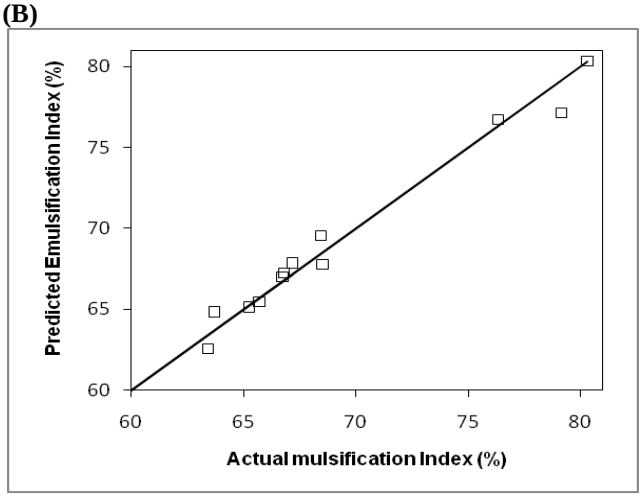
R^2= 0.9829 R^2(adjusted)= 0.9676

As can be observed in the plots, actual values are distributed near to the straight lines which indicated a high dependence between the observed and the predicted values of responses and that the regression models fit the experimental data quite well. So, it can substantiate the results which were achieved from the values of determination coefficients.

(A)



(B)



(C)

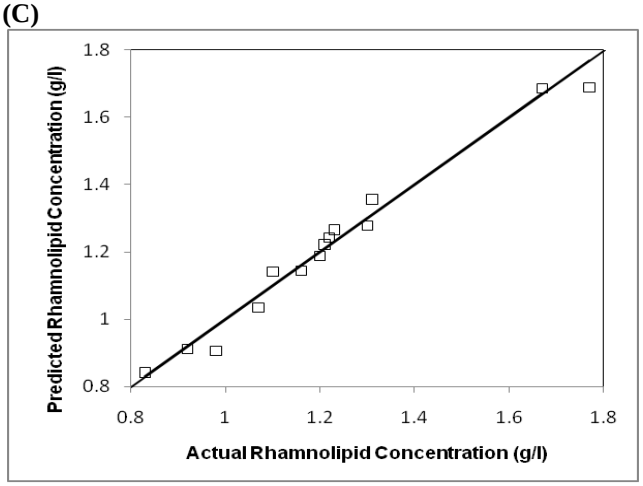


Figure 2. Experimental vs. Predicted values for (A): Surface Tension, (B): Emulsification Index, and (C): Rhamnolipid Yield.

Taking into account the whole parameter estimate and the corresponding P values, it can be suggested that, among the test variables, agitation rate, filling volume, and aeration rate are respectively the most influential parameters in biosurfactant production in a shaken bioreactor. Moreover, solving the regression equations, the optimal values of the test variables, within the explored experimental domain, were achieved in coded units, as follows: *X1*= -1, *X2*= 0.84, and *X3*= 1 which means that the best results would attain when selecting the following conditions:

filling volume=25 ml, agitation rate=292 rpm, and aeration rate= 1vvm. These results reveal that a high aeration rate coupled with an almost high agitation rate, besides a low filling volume tends to give acceptable yields.

Furthermore, the best results that can be achieved under the optimum conditions, according to the models' predictions, for surface tension, emulsification index, and rhamnolipid yield are 29.111 mN/m, 80.092%, and 1.857 g/L, respectively, which experimentally confirmed with a deviation less than 5%.

Interpretation of contour plots

The entire relationships between factors and responses can be better understood by examining the planned series of contour plots generated based on the predicted models (Eqs. 1-3) that illustrated graphically the responses values vs. the levels of variables. Such plots are helpful in studying the effects of the factors variations in the studied domain, and consequently, in determining the optimal experimental conditions.

Since the effects of variables on the three examined responses are almost similar, just the contour plots for the rhamnolipid yield (R3) are presented in this section (Fig.3 (A-C)). The highest points in these plots indicate the optimal operating conditions, attaining the most rhamnolipid yield. Figure 3(A) represents the three dimensional contour plot between the rhamnolipid yield as the response and the combined effect of filling volume and agitation rate, while the level of the aeration rate is fixed at its optimum value (1vvm). It can be observed that both of the filling volume and agitation rate have definite influence on biosurfactant production. Biosurfactant yield is drastically declined at high filling volumes, whereas the production yield is consistently increased by increasing shaking frequency. Agitation rate of 335 rpm is shown to be the best concentration for the maximum production of rhamnolipid, because the production gradually diminished at higher rates of agitation. Higher rotary velocities result in increasing the oxygen mass transfer efficiency to the aqueous medium and yielding better microbial growth and therefore more biosurfactant formation from aerobic bacterium *Pseudomonas aeruginosa* MM1011.

However, the yield reduction in much higher velocities can be attributed to the heavy foaming caused by emulsification of rhamnolipid during vigorous shaking that can reduce the oxygen mass transfer to the medium and therefore decrease the yield of rhamnolipid production. Thus, high rhamnolipid production requires choosing low levels of filling volume and almost high levels of agitation rate. It should also be noted that, as obviously indicated in the plot, the filling volume is more influential than agitation rate in rhamnolipid production.

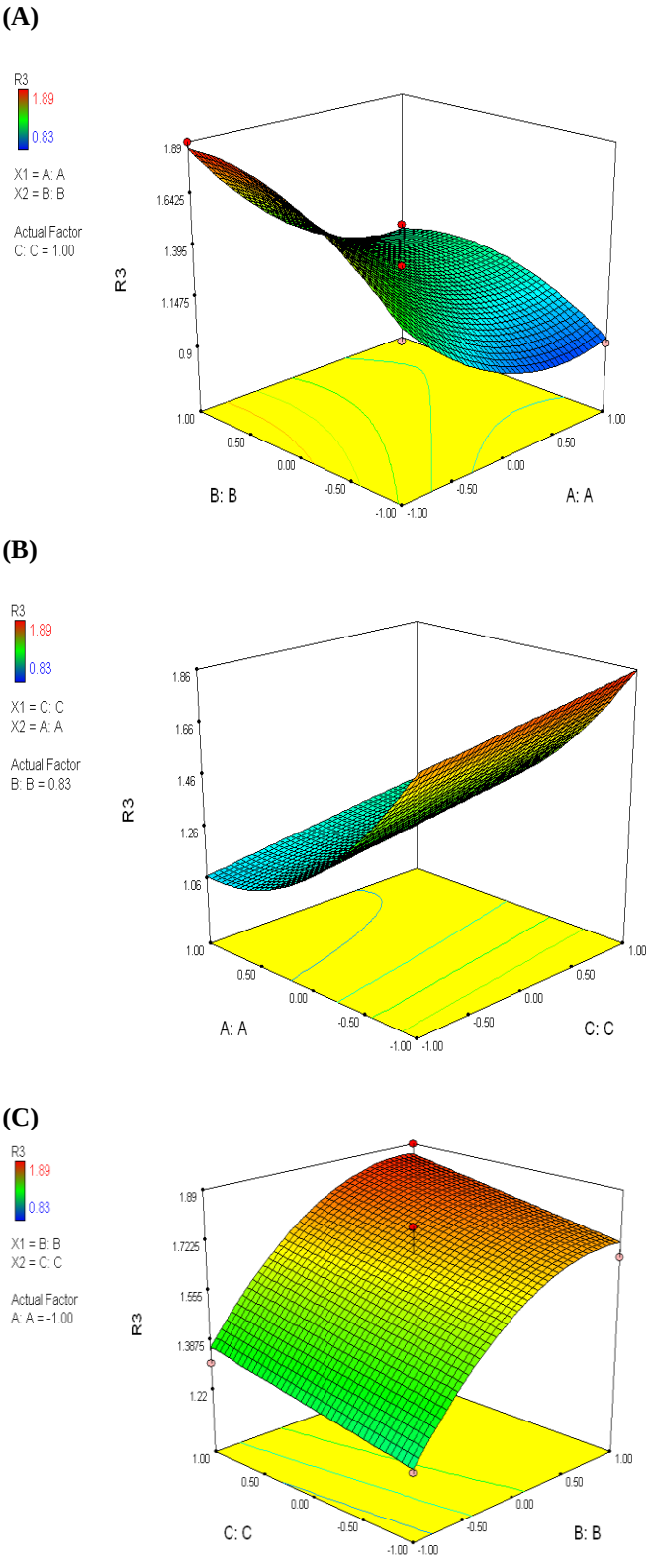


Figure 3. Three dimensional contour plots for the maximum biosurfactant production as a function of (A): filling volume and agitation rate, (B): filling volume and aeration rate, and(C): agitation and aeration rates

The joint effect of filling volume and aeration rate is depicted in Figure 3 (B), with agitation rate fixed at 292 rpm. According to this figure, the filling volume affects the rhamnolipid production more significantly than aeration rate. Furthermore, as can be seen in the figure, the aeration rate has a positive relationship with rhamnolipid yield, while increase in filling volume leads to a noticeable decrease in rhamnolipid yield. Since, in high filling volumes, by growing the microbial population, the oxygen uptake rate by microorganisms gradually exceeds the oxygen transfer rate to the medium. Consequently, oxygen limitation occurs in the medium, which in turn can manifestly reduce the production yield.

In a similar pattern of the previous figures, Figure 3 (C) illustrates the mutual effect of agitation and aeration rates on rhamnolipid yield at fixed filling volume of 25 ml. It can be verified in the figure that the strain showed maximum rhamnolipid production close to the high level of agitation rate. Moreover, like the joint effect of filling volume and aeration rate, the effect of agitation rate is more considerable than that of aeration rate and a definite rise transpires by increasing the aeration rate.

Biosurfactant production under optimized conditions

To check the experimental design results, subsequent batch fermentation was carried out selecting the optimal processing conditions (filling volume = 25 ml, shaking frequency = 292 rpm, and aeration rate = 1vvm), and different process parameters such as growth, surface tension, biosurfactant concentration, glucose consumption and emulsification activity were recorded, at regular intervals, during 168 h of cultivation (Fig. 4).

The curve of rhamnolipid production shows that there was a little rhamnolipid production during the early and mid-exponential phase, and it was mainly produced by the end of the exponential phase and at stationary phase, which is completely consistent with previous findings [10, 36], that reported rhamnolipid as a secondary metabolite secreted by *P. aeruginosa* in stationary phase of growth. Moreover the value obtained for maximum yield of production (1.68 g/L), is more than what reported [7, 37], that indicates the promising fermentation conditions which were applied in this study.

The surface tension of virgin culture medium was measured as 70.34 mN/m. As can be observed in the Figure 4, the surface tension of the culture medium dropped to about 30 mN/m during the exponential phase of growth (in which the rhamnolipid production was low) and almost remained constant afterward. This reveals that rhamnolipid produced by *Pseudomonas aeruginosa* MM1011, was a potent biological surface active compound and its concentration after 120 h was high enough for micelle formation and reaching critical micelle concentration (CMC) point. In addition to surface tension, emulsification activity of the culture, demonstrated high development during the exponential and stationary phases, but as it can be observed, compared with surface tension, emulsification activity needed more time for growth and rhamnolipid production, in order to reach its maximum point. Eventually, the glucose consumption curve displays the constant that after 6 days of incubation the glucose supply in the medium completely depleted that it shows the ideal growth conditions without any oxygen limitation.

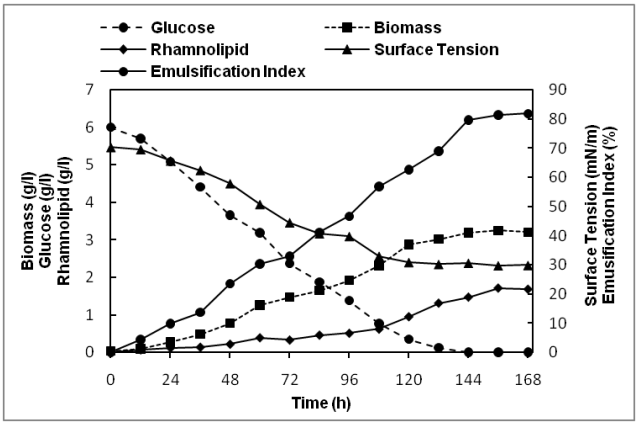


Figure 4. Fermentation of *Pseudomonas aeruginosa* MM1011 during 168 h, under optimized conditions.

Conclusion

In conclusion, RSM was proven to be an invaluable tool to optimize rhamnolipid production by *P. aeruginosa* MM 1011, and the presented models could predict responses in good agreement with experimental results. Also, the results indicated that all of the three factors were effective parameters in biosurfactant production and the surface tension, rhamnolipid production and emulsification activity under optimum conditions, were measured 31.00 mN/m, 1.89 g/L and 80.23 %, respectively. Furthermore, the preliminary results confirmed that the miniaturized shaken flasks can potentially be adapted for rhamnolipid production, and perhaps further developed, for large-scale production.

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