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OPTIMIZATION OF A BACTERIAL BIOSURFACTANT PRODUCTION

OPTIMIZACIÓN DE LA PRODUCCIÓN DE UN BIOSURFACTANTE BACTERIANO

Ma. A. Martínez-Trujillo, I. Membrillo Venegas, S.E. Vigueras-Carmona, G. Zafra-Jiménez and M. García-Rivero*

División de Ingeniería Química y Bioquímica, Tecnológico de Estudios Superiores de Ecatepec, Av. Tecnológico s/n Col. Valle de Anáhuac CP 55210, Ecatepec, Edo. de México, México.

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Abstract

Bacterial biosurfactant production was optimized by means of Response Surface Methodology (RSM), in which nitrogen and carbon concentrations, as long as the addition of an immiscible substrate, supplemented for increasing the efficiency of biosurfactant biosynthesis, were the evaluated variables. A mixture of yeast extract-$NaNO_3$, and fructose was used as nitrogen and carbon source, respectively. Under the assayed conditions yeast extract-$NaNO_3$ relationship and hexadecane concentrations were the factors which had a significant effect on biosurfactant production at flask level. The optimal conditions, estimated by the canonical analysis of the corresponding response surface were used at a 600 mL bioreactor, obtaining a biosurfactant production measured as 74.23 % of emulsification index, which was similar to the estimated by de quadratic model.

Keywords: response surface methodology; canonical analysis; nitrogen and carbon source concentration.

Resumen

Se optimizó la producción de un surfactante bacteriano por medio de la Metodología de Superficie de Respuesta (MSR), como variables de estudio para incrementar la eficiencia de biosíntesis del biosurfactante se evaluaron las concentracines de carbono y nitrógeno, así como la adición de un sustrato insoluble. La fuente de nitrógeno fue una mezcla de extracto de levadura- $NaNO_3$ y como fuente de carbono se usó fructosa. Bajo las condiciones ensayadas la relación extracto de levadura- $NaNO_3$ y la concentración de hexadecano fueron los factores que tuvieron un efecto significativo en la producción del biosurfactante a nivel matraz. Por medio del análisis canónico se estimaron las condiciones óptimas de la superficie de respuesta, que fueron usadas en un biorreactor de 600 mL, en el cual se obtuvo una producción de biosurfactante de 74.23% medido por el índice de emulsificación, cuyo valor fue similar al estimado por el modelo cuadrático.

Palabras clave: metodología de superficie de respuesta; análisis canónico, concentración de la fuente nitrógeno y de carbono.

1 Introduction

The worldwide use of surfactants has grown enormously over the past few decades because they have been commonly used in the petroleum, food, and pharmaceutical industries as emulsifiers and wetting agents (Shing *et al*., 2007). However, the increasing environmental concerns about chemical surfactants have triggered attention to biosurfactants essentially due to their biodegradable nature, low toxicity, and stability at relatively high temperature and adverse environments (Fakruddin 2012; Helmy *et al*., 2011). Because of these characteristics, many companies which use chemical surfactants in their processes are now looking to replace some or all of them with biosurfactants (Marchant and Banat, 2012 Helmy *et al*., 2011).

Nevertheless, several problems should be solved before more widespread use can be envisaged. These problems are related to their low yield and high cost of production, including downstream processing and also the tailoring of the molecules to specific applications (Marchant and Banat, 2012; Nitschke and Costa, 2007).

The cost of biosurfactant production can be reduced by selecting an efficient strain, optimizing

*Corresponding author. *E-mail*: mayolagariv@yahoo.com.mx

medium composition or by using alternative inexpensive substrates (Rodrigues *et al*., 2006a). About medium composition, the carbon and nitrogen sources have shown significant effects on the quality, quantity, and cost of the resulting biosurfactants. Several reports have shown that carbohydrates are the most suitable carbon source to promote synthesis of biosurfactants in some microorganisms (Fakruddin 2012; Abouseoud *et al*., 2008; Rahman and Gakpe, 2008) meanwhile inorganic nitrogen source are preferred by microorganism, but in restricted conditions (Abouseoud *et al*., 2008; Onwosi and Odibo, 2012; Saikia *et al*., 2012).

Some studies have focused on the synergistic effects of insoluble carbon sources like vegetal oils, motors oils, diesel and hydrocarbons over the efficiency and biosynthesis of biosurfactants (Makkar and Cameotra, 2002; Rahman and Gakpe, 2008; Calvo *et al*., 2008). It has been demonstrated that microorganisms release biosurfactants to facilitate the uptake of hydrophobic compounds by solubilization and emulsification (Abdel-Mawgoud *et al*., 2010). Thus, they can stimulate the growth of hydrocarbon degrading microorganisms, improving their capacity to utilize these compounds.

One of the most accepted methodologies used for optimizing medium composition is the response surface methodology, which has been used widely for parameter optimization of the process, due its easy operation. The response surface methodology, or MSR, is a collection of mathematical techniques and useful statistics used in modeling and analysis of problems in which the response of interest is influenced by several variables and where the objective is to optimize this response (Montgomery, 2006). This statistical technique has been successfully utilized to optimize medium composition for the synthesis of metabolites and the biodegradation processes of some contaminant (Corona-González *et al*., 2013, Tepe and Dursun, 2014; Abbasi *et al*., 2012; Gomez and Sartaj, 2014; Huang *et al*., 2013), to name a few applications.

The objective of the present work was to optimize the biosurfactant production of a bacterial strain in batch fermentation applying the response surface methodology to three independent variables: nitrogen and carbon source, as long as immiscible substrate concentrations

2 Methods

2.1 Microorganism

A Gram positive bacterial strain, isolated from petroleum contaminated site (García-Rivero, 2007), was used for biosurfactant production assays in liquid culture. The bacterial strain was stored in tripticase soy agar slants at 4°C and sub-cultured every four weeks. Two loops of culture slant were inoculated in tripticase soy broth and incubated at 30°C and 150 rpm for 3 days. Afterwards, the cells were centrifugated at 15,000 rpm for 15 min, washed twice with physiological saline solution (NaCl, 0.9% w/v) and re-suspended in the mineral medium. The microbial suspension was incubated at 30°C and 150 rpm by 24 h and the resulting inoculum suspension was standardized to 1.3 optical density units, measured at 480 nm. Thus, considering the standard curve for cell concentration versus optical density, the inoculum had a biomass concentration of 0.1 g L^{-1}.

2.2 Medium and cultivation

A simple medium consisting of mineral salts (García-Rivero, 2007) was used for the inoculum preparation and optimization assays, according specifications given in each case. The basal composition, in g.L^{-1} was: KH_2PO_4, 1; KCl, 0.5; $Mg2SO_4{\cdot}7H_2O$, 0.25 and 2 mL of mineral solution. The mineral solution (%, w/v) contained the following: $FeSO_4{\cdot}7H_2O$, 0.1; $CuSO_4{\cdot}5H_2O$, 0.015; $ZnSO_4{\cdot}7H_2O$, 0.161 and $MnSO_4{\cdot}7H_2O$, 0.008. The concentration of nitrogen and carbon sources, and immiscible substrate, were adjusted according to the corresponding experiment design. The microorganism was cultured in 250 mL Erlenmeyer flasks containing 100 mL of mineral medium, which were inoculated with 10% (V/V) of inoculum suspension. The flasks were incubated at 150 rpm and 30 °C by 5 d.

2.3 Central composite design

Central composite design is one of the most important experimental designs used in process optimization studies for the construction of a quadratic response surface model (Montgomery, 2006). It consists of a two-level full factorial design superimposed on a star design that is augmented by additional centre points. The centers of the two designs coincide. This is the last step of the response surface methodology, and is used when the interaction among factors

results significant for the response, as it means that the production is around the optimum response vicinity. So, the resulting quadratic model of this design can show the exact conditions in which the highest response would be obtained, by means of the corresponding canonical analysis (Palasota y Stanley, 1992).

Based on the investigation results of biosurfactants production by various microorganisms (Calvo *et al*., 2009) we assumed as the most important factors for the biosurfactant production were the concentration of carbon and nitrogen sources and the presence of an immiscible substrate. Accordingly, the effects of fructose (F), yeast extract- $NaNO_3$ relationships (YE-SN) and *n*-hexadecane (H) concentrations were used at the central composite design composed by three variables (k = 3), six replicates at the central point (CP = 6), and six experiments at the axial point (2*k = 6), resulting in 20 experiments. The axial distance α was chosen to be 1.681 to make this design rotable.

The levels of these independent variables are shown in Table 1. As the dependent variable we used the measured emulsifying index (EI_{24}), which reflected indirectly the amount of biosurfactant produced in each case. This experimental design was developed at flask level in independent experiments.

2.4 Statistical analysis

Data from the central composite design were used for fitting the regression coefficients of a second-order model. The quality of fit model regression was expressed by the coefficient of determination R^2, and the statistical significance of its parameters was checked by the analysis of variance. The significance of the regression coefficient of the second-order model was tested by a t-test. The level of significance was given as values of Prob > F less than 0.01. After the elimination of non significant parameters, an equation that represents the effect of the significant variables in the biosurfactant production was obtained.

Table 1. Experimental design matrix

Run	Coded variables			Reals variables		
	(X1) F	(X2) YE-SN	(X3) H	(X1) F (%)	(X2) YE-SN (g L^{-1})	(X3) H (g L^{-1})
1	-1	-1	-1	1	3, 0	1
2	1	-1	-1	3	3, 0	1
3	-1	1	-1	1	0, 3	1
4	1	1	-1	3	0, 3	1
5	-1	-1	1	1	3, 0	5
6	1	-1	1	3	3, 0	5
7	-1	1	1	1	0, 3	5
8	1	1	1	3	0, 3	5
9	-1.681	0	0	0.3	1.5, 1.5	3
10	1.681	0	0	3.7	1.5, 1.5	3
11	0	-1.681	0	2	4.05, 0	3
12	0	1.681	0	2	0, 4.05	3
13	0	0	-1.681	2	1.5, 1.5	0
14	0	0	1.681	2	1.5, 1.5	6.4
15	0	0	0	2	1.5, 1.5	3
16	0	0	0	2	1.5, 1.5	3
17	0	0	0	2	1.5, 1.5	3
18	0	0	0	2	1.5, 1.5	3
19	0	0	0	2	1.5, 1.5	3
20	0	0	0	2	1.5, 1.5	3

Fructose concentration, F

Yeast extract- $NaNO_3$ relationship concentration, YE-SN

n-hexadecane concentration, H

2.5 Determination of the optimal operating conditions

Assuming non-significant lack of fit of the second-order model, it was used to determine the location and the nature of the stationary point of the fitted surface by means of canonical analysis (Rodrigues *et al*., 2006). Accordingly, the analysis of the fitted surface in the stationary point allowed to obtain the optimum operational conditions, and under these conditions was possible to predict the biosurfactant production (Montgomery, 2006).

2.6 Emulsification index

The culture broth obtained in each experiment was thermally treated (115°C for 15 min) and used to measure the emulsification activity by the Cooper method (Cooper and Goldenberg, 1987) with diesel fuel as the substrate for emulsification. Six milliliters of the substrate were added to four milliliters of the culture broth and the mixture was shaken for 2 min. The emulsification index (EI_{24}) was determined after 24 h as the ratio between the height of the emulsion layer and the total height of the liquid column, expressed in percent

3 Results and discussion

3.1 Experimental design: biosurfactant production

The results of the central composite design are presented in Table 2. Biosurfactant production showed a considerable variation determined by the independent variables used in the design. There was no production of biosurfactant in experiment 11 and 12, in which only YE or SN were used. However, the maximum biosurfactant production (76%) was obtained in treatment runs 15-20, localized at the central point, in which both nitrogen sources were used in the same proportion. It is important to highlight that sodium nitrate and yeast extract have been reported as the best substrate for biosurfactant production (Fakruddin, 2012; Dastgheib *et al*., 2008).

Table 2. Results of biosurfactant production, measured as EI_{24}, under different experimental conditions according to the central composite design matrix

Runs	F (%)	YE-SN (g L^{-1}°)	H (g L^{-1})	EI_{24} (%)
1	1	3, 0	1	17
2	3	3, 0	1	36
3	1	0, 3	1	3.1
4	3	0, 3	1	4.6
5	1	3, 0	5	30.1
6	3	3, 0	5	51
7	1	0, 3	5	36
8	3	0, 3	5	22
9	0.3	1.5, 1.5	3	40
10	3.7	1.5, 1.5	3	40
11	2	4.05, 0	3	0
12	2	0, 4.05	3	0
13	2	1.5, 1.5	0	54
14	2	1.5, 1.5	6.4	55
15	2	1.5, 1.5	3	70.17
16	2	1.5, 1.5	3	70.17
17	2	1.5, 1.5	3	73.68
18	2	1.5, 1.5	3	73.68
19	2	1.5, 1.5	3	73.68
20	2	1.5, 1.5	3	76

Fructose concentration, F

Yeast extract- $NaNO_3$ relationships concentration, YE-SN

n-hexadecane concentration, H

Results showed that high concentration of organic or inorganic nitrogen source had a negative effect on biosurfactant production; but when both nitrogen sources were added at equal concentration the biosurfactant production increased. A similar result was reported by Abbasi *et al*. (2012) during the production of biosurfactants by *Pseudomonas aeuroginosa*, they showed the synergism of sodium nitrate and yeast extract on the biosurfactant production. However, in biosurfactant production the yeast extract optimum concentration to be employed was organism and culture medium dependent (Fakruddin 2012). Also it was demonstrated that the addition of yeast extract has a positive effect on biosurfactant production by Candida ingens (Amézcua-Vega *et al*., 2007) and *Corynebacterium fascians* (Cooper *et al*., 1981).

On the other hand, fructose has been considered an effective carbon substrate to produce biosurfactant by *P. aeuroginosa* (Abbasi *et al*., 2012) which explains why the biosurfactant is produced efficiently in the presence of fructose; however, in this case fructose seemed not to be significant for the biosurfactant

production (Table 2). Furthermore, the addition of water insoluble substrate, in this case hexadecane, promotes the microbial synthesis of biosurfactants (Calvo *et al*., 2009), because hydrocarbons are hydrophobic compounds which induce bacterial cell to produce biosurfactants in order to improve the solubility of these substrates (Abbasi *et al*., 2012; Beal and Betts, 2000).

3.2 Statistical analysis of the response

The regression analysis of the experimental data produced a second order-model to explain the biosurfactant production, considering T<0.05 (Table 3) the model that describes the dependence of biosurfactant production by the factor is shown in the following equation:

$$Y = 72.8637 - 4.9637(X2) + 5.8127(X3) - 26.1853(X2)^2 - 7.3272(X3)^2 \quad (1)$$

Where Y is the studied response (biosurfactant production); $X2$ and $X3$ are the yeast extract- $NaNO_3$ relationships and n-hexadecane concentrations, respectively.

The Student t-distribution and the resultant P-value, along with the parameter estimated, are given in Table 3. The results obtained shows that, only the independent variables X2 and X3 have a significant effect on biosurfactant production. The negative coefficient for X2 shows a linear effect to decrease biosurfactant production, while positive coefficient for X3 indicated a linear effect to increase studied response. The quadratic term of X2 and X3 also have a significant effect. However, none of the interactions among the three variables were found to be significant to the variable response.

The analysis of variance (ANOVA) of the model is shown in Table 4, in this it is evident that the model was highly significant, as suggest by the model F value and low probability value ((P model>F) = 0.0001). The ANOVA showed that the model explains 94% of the variability in the data, and only about 6% of biosurfactant production was not attributed to the independent variables. Therefore the quadratic model was used to build response surface.

The response surface obtained (Figure 1) shows the joint effect of hexadecane and yeast extract-$NaNO_3$ relationship concentrations on the biosurfactant production. It can be seen that the yield of the biosurfactant production, measured as IE(%), will be maximum around the center point values of the codified variables X2 and X3, corresponding to 1.5 gL^{-1} of yeast extract and $NaNO_3$, and 3 $gL^{-1}$of hexadecane. According to the above, an increase or decrease of values of variables will produce a diminution on biosurfactant production.

Table 3. The least- square fit and parameters (significant of regression coefficients)

Model term	Degree of freedom	Estimate	Standard error	*t*-value	P> |*t*|
Intercept	1	72.8637	4.03	18.076	0.0001
x1	1	1.9883	2.42	0.819	0.4342
x2	1	-4.9637	2.42	-2.043	0.0714
x3	1	5.8127	2.42	2.393	0.0404
x1x2	1	-6.55	3.18	-2.054	0.0701
x1x3	1	-1.7	3.18	-0.533	0.6068
x2x3	1	2.775	3.18	0.87	0.4067
x1x1	1	-12.3445	2.4	-5.137	0.0006
x2x2	1	-26.1853	2.4	-10.898	0.0001
x3x3	1	-7.3272	2.4	-3.049	0.0138

Table 4. Analysis of variance (ANOVA) from the central composite design

Source	Degree of freedom	Sum of squares	Mean square	F-value	P> F
Model	9	11990.091	1332.23241	16.383	0.0001
Error	9	731.8704	81.31893		
C Total	18	12721.9621			
	CV	22.76895		R-square	0.9425
				Adj RS sq	0.8849

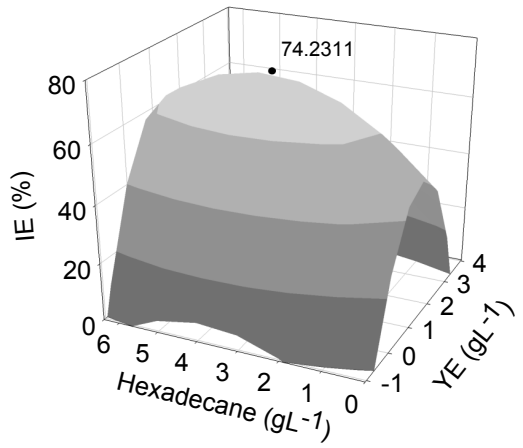

Fig. 1. Response surface plot of the combined effects of hexadecane concentration and Yeast extract-NaNo3 relationship concentration on the biosurfactant production.

The high biosurfactant production at the central point is attributable to the operational conditions determined by independent variables: the presence of insoluble substrate (hexadecane) that promotes biosurfactant production, and the enrichment of the medium with yeast extract, that has demonstrated to have an important effect on biosurfactant production in different microorganism (Amézcua-Vega *et al*., 2007; Rodrigues *et al*., 2006b).

There are controversial effects of the carbon source on biosurfactant production due to this production is dependent on microorganisms and culture conditions. Using hydrocarbons as the sole carbon source usually results in a null biosurfactant production (Abdel-Mawgoud *et al*.; Joshi *et al*., 2008), although other works indicated a positive effect when an insoluble substrate was used: vegetal oils, diesel, hydrocarbons (Calvo *et al*., 2008); furthermore, in the case of B. subtilis the combination of a water soluble carbon source (sucrose) and a hydrocarbon (*n*-hexadecane) did not have a negative effect on the biosurfactant production (Pereira, *et al*., 2013; Gudiña *et al*., 2012). In this work a positive effect was observed when fructose and *n*-hexadecane was added, that in our knowledge has not been reported.

In order to find the stationary point for biosurfactant production (i.e. conditions that allow producing the maximum biosurfactant production), a canonical analysis was developed. The model was written as matrix notation and the resulting matrix was solved to obtain the coefficient values and subsequently the real values of the studied variables that produce the maximum biosurfactant yield (Palasota and Stanley, 1992). The results were: X1 = 2.07 % of fructose, X2 = 1.62 and 1.37 $gL^{-1}$of yeast extract and $NaNO_3$, respectively, and X3 = 3.74 gL^{-1} of hexadecane. It should be noted that coefficient for X2 in the regression model suggested that yeast extract- $NaNO_3$ relationships should decrease in order to obtain a higher biosurfactant production, i.e. it was necessary to low the concentration of the yeast extract in this mixture. The canonical analysis provided the yeast concentration that could ensure the highest biosurfactant production.

From the codified values of X1, X2 and X3 at their maximum points, a theoretical biosurfactant production of 74.23 % of IE was determined. In order to verify that this yield was possible to obtain, a duplicated experiment was carried out in a 600 mL bioreactor, in which the basal medium was modified according to the concentrations listed above to the studied variables. The IE% obtained in this case was 74 ± 2.82 %. This value had a 2% difference from the predicted value, discrepancy that could be explained by the slight variation in experimental conditions.

Conclusions

Using the central composite design and response surface analysis was possible to find out the optimal operation conditions to obtain maximum biosurfactant production. Under the assayed conditions, yeast extract-$NaNO_3$ relationship and hexadecane concentrations were the factors which have a significant effect on biosurfactant production. The predicted and verifiable biosurfactant production under optimal conditions in shake flasks experiments was 74 and 74 ± 2.82 %, respectively.

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