

Full Length Research Paper

Statistical optimization of low-cost medium for economical production of *Bacillus subtilis* biosurfactant, a biocontrol agent for the olive moth *Prays oleae*

Dhouha Ghribi^{1,2*}, Ines Mnif^{1,2}, Hanen Boukedi³, Radhouane Kammoun² and Semia Ellouze-Chaabouni¹

¹Unité Enzymes et Bioconversion, National School of Engineers of Sfax, Tunisia.

²Higher Institute of Biotechnology of Sfax, Tunisia.

³Laboratory of Biopesticides, Centre of Biotechnology of Sfax, Tunisia.

Accepted 11 November, 2016

Biosurfactants are currently not a feasible alternative to chemically synthesized surfactants as a result of their potentially high production costs. In this work, *Bacillus subtilis* SPB1 biosurfactant was shown to be efficient in the biocontrol of the olive moth *Prays oleae*. Its production was improved by optimizing the medium components using inexpensive substrates. The effect of orange peels, soya bean and diluted sea water on SPB1 biosurfactant production was studied and was adjusted using central composite design. The experimental results were fitted to a second-order polynomial model that yielded a determination coefficient of $R^2=0.932$. The optimal medium for biosurfactant production was found to be composed only by orange peels (15.5 g/L), soya bean (10 g/L) and diluted sea water (30%). The predicted and observed response were 4.3 g/L (with desirability = 0.21) and 4.45 g/L, respectively. In comparison to original level production, two fold increases had been obtained.

Key words: *Bacillus subtilis*, biosurfactant, central composite design, optimization, *Prays oleae*.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and the interface, respectively (Banat et al., 2010). They are a structurally diverse group of surface-active molecules synthesized by microorganisms (Lu et al., 2007) Rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis*, emulsan from *Acinetobacter calcoaceticus* and

sophorolipids from *Candida bombicola* are some examples of microbial-derived surfactants. The reason for their popularity as high value microbial products is primarily because of their specific action, low toxicity, high biodegradability, effectiveness at extremes temperature, extreme pH and high salinity, widespread applicability and their unique structures which provide new properties that the classical surfactants may lack (Desai and Banat, 1997). Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial cultivation process using cheaper agro-based substrates and waste materials (Makkar and Cameotra, 2002). Despite many advantages of surfactins over chemical agents and great recent advances in our understanding of surfactins (Eeman et al., 2006; Heerklotz et al., 2007), there have

*Corresponding author. E-mail: dhouhag@yahoo.fr. Tel: 21674674364. Fax: 21674675055.

been hardly any significant applications of surfactins, mainly because of the low strain productivity and the need for expensive substrates (Mukherjee et al., 2006). Efforts must therefore be redirected to improve production efficiency and recovery bioprocesses in order to optimize yields (Mukherjee et al., 2006).

To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering processes and the use of cost-free or cost credit feedstock for microorganism growth and surfactant production (Maneerat, 2005). The choice of inexpensive raw materials is important to the overall economy of the process because they account for 50% of the final product cost and also reduce the expenses with wastes treatment (Rahman et al., 2002). In this investigation, we tried to optimize cultivation media for low-cost production of biosurfactants based on isolated *B. subtilis* strain using response surface methodology (RSM). The use of experimental factorial design and RSM has already been successfully applied in other fields and is well adapted to the study of the main effects and the interaction effects of the factors on the biosurfactant production. The conventional method of optimisation involves varying one parameter at a time and keeping the others constant. This does not often bring about the effect of interaction of various parameters as compared to factorial design (Griffin et al., 1992). In the present work, we have attempted to optimize the production of biosurfactant from *B. subtilis* SPB1 using central composite design.

MATERIALS AND METHODS

Microorganism

Biosurfactant producing bacterium was isolated in our laboratory from Tunisian soil and it was identified as *B. subtilis* SPB1 (HQ392822) by morphological, biochemical and 16S Ribosomal deoxyribonucleic acid (rDNA) sequence analysis (Ghribi and Ellouze-chaabouni, 2011). This strain was used for large scale biosurfactant-production studies because of its large spectrum of bioactivity which has a great potential for biotechnological and pharmaceutical applications.

Inocula preparation

The inocula were prepared as follows: one isolated colony was dispensed in 3 ml of LB medium and incubated overnight at 37°C. Aliquots (0.2 ml) were used to inoculate 250 ml Erlenmeyer flasks containing 50 ml LB medium. After 6 h of incubation at 37°C in a rotary shaker set at 200 rpm, the absorbance at 600 nm was determined. The culture broth was used to inoculate the studied media to start with an initial optical density of 0.15, corresponding to almost 1.2×10^7 CFU/ml.

Orange peels, soya bean meal and sea water sources

Peelings of Tunisian sweet orange variety containing 36% sugar, 6% proteins and 2% lipid were dried and thinly crushed.

Commercial soya bean, containing 44% proteins, 26% sugar and

7% lipid was obtained from a local mill of animal meals (ALCO Affes Group, Sfax, Tunisia). Sugar and protein contents of orange peels and soya bean meal were determined according to the methods described, respectively, by Dubois et al. (1956) and Pearson (1970). The lipids determination was carried out by continuous extraction using Soxhlet apparatus (AOAC, 1984). Sea water was sampled from the Mediterranean-sea coast of Sfax (Tunisia). It contains (g/L): Na^+ , 12; Cl^- , 22; K^+ , 0.4; Ca^{2+} , 0.14; HCO_3^- , 0.40; Mg^{2+} , 1.3; SO_4^{2-} , 2.640; Fe^{2+} ; Mn^{2+} (Ghribi et al., 2007).

Selection of a suitable substrate

Various industries by-products like potato peels, chickpea flour, wheat its, wheat flour, millet, soya bean meal, barley flour, orange peels, banana peels, barley its, rice flour and corn starch were screened for biosurfactant production. Sterilized bran was used as substrate at a concentration of 40 g/L into media containing 6 g/L urea and 20% sea water (Ghribi et al., 2007). The substrate yielding the maximum biosurfactant production was identified and selected for further studies using RSM.

Culture conditions

Culture was incubated for 48 h at 37°C, pH 7 on a rotary shaker set at 150 rpm into 250 ml erlenmeyer flask containing 50 mL of medium (Ghribi and Ellouze-Chaabouni, 2011). Production medium was composed of orange peels, soya bean and diluted sea water at concentrations mentioned with results. All experiments were performed in triplicate.

Extraction of crude biosurfactant

The crude biosurfactant was isolated from the cell free broth of 48 h grown culture. The bacterial cells were removed from surfactant containing culture broth by centrifugation at 10.000 rpm at 4°C for 20 min. The supernatant was precipitated overnight at 4°C by adding concentrated HCl to achieve a final pH of 2.0, to precipitate lipids and proteins. Grey white pellets formed by precipitation were collected by centrifugation at 10.000 rpm at 4°C for 20 min. The pellets corresponding to the crude surfactant were weighted for quantification after desiccation at 105°C for 24 h (Ghribi and Ellouze-Chaabouni, 2011). Culture without inoculation was used as a negative control to take account possible contribution of lipids and proteins from substrates. The negative control was included in each experiment and each cultural condition. Crude biosurfactant weight was calculated as the result of subtracting the grey white pellet weight obtained with the negative control from that measured with the culture containing the biosurfactant producing strain. The values presented are the average of the results of three determinations of two separate experiments for each cultural condition.

Response surface methodology

RSM is an empirical modeling technique used to evaluate the relationship between a set of controllable factors and observed responses. RSM was used to determine the optimum components of culture medium to maximize biosurfactant synthesis. The experimental design chosen for this study was the central composite design for three independent variables within the region of three dimensional observation spaces allowing a minimum number of experimental runs. It was generated using NemrodW Version 2007 software (LPRAI, Marseille, France). Each variable was analyzed at five levels coded as $-\alpha$, -1, 0, +1 and $+\alpha$ (Table 1). A set of 20 runs including 2^3 full factorial design experiments (runs N° 1 to 8),

Table 1. Design of experiment and response of the central composite design for the production of biosurfactant using *B. subtilis* SPB1.

Run N°	Orange peels (g/L) (x_1) X1	Soya bean (g/L) (x_2) X2	Sea water (%) (x_3) X3	Biosurfactant (g/L) experimental values	Biosurfactant (g/L) calculated values
1	(-1) 15	(-1) 15	(-1) 20	4.30	4.32
2	(1) 45	(-1) 15	(-1) 20	3.00	3.31
3	(-1) 15	(1) 45	(-1) 20	2.68	2.86
4	(1) 45	(1) 45	(-1) 20	3.80	3.92
5	(-1) 15	(-1) 15	(1) 40	3.60	3.68
6	(-1) 15	(-1) 15	(1) 40	2.90	2.91
7	(-1) 15	(1) 45	(1) 40	2.50	2.39
8	(1) 45	(1) 45	(1) 40	3.50	3.68
9	(-1.682) 4.77	(0) 30	(0) 30	3.50	3.48
10	(+1.682) 55.23	(0) 30	(0) 30	4.00	3.72
11	(0) 30	(-1.682) 4.77	(0) 30	3.80	3.64
12	(0) 30	(+1.682) 55.23	(0) 30	3.20	3.06
13	(0) 30	(0) 30	(-1.682) 13.18	4.00	3.71
14	(0) 30	(0) 30	(1.682) 46.82	3.00	2.98
15	(0) 30	(0) 30	(0) 30	4.30	4.25
16	(0) 30	(0) 30	(0) 30	4.40	4.25
17	(0) 30	(0) 30	(0) 30	4.10	4.25
18	(0) 30	(0) 30	(0) 30	4.22	4.25
19	(0) 30	(0) 30	(0) 30	4.15	4.25
20	(0) 30	(0) 30	(0) 30	4.3	4.25

x represent the coded level of variables; X represent the actual level of variables. Figures in parentheses denote coded level of variables.

six axial points (runs N° 9 to 14) and six center points (runs N° 15 to 20) was carried out. The model evaluates the effect of each independent variable on the response. The mathematical relationship of the independent variables and the response can be calculated by the quadratic polynomial equation:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3$$

Where X1, X2 and X3 are the coded factors studied. The significance of the coefficients is evaluated by multiple regression analysis based upon the *F*-test with unequal variance ($P < 0.05$) (Nair and Panda, 1997). The computation was carried out by multiple regression analysis making use of the least squares method. The significant selected components of the medium were orange peels, soya bean meals and sea water based on Plackett Burman study (data not shown). The model coefficients were estimated using multi linear regression. The six replicates at the center point of the design permitted to estimate the variability of the experimental results. The compatibility of the proposed model with the obtained experimental data was verified by performing the analysis of variance. The statistical model was validated with respect to all of the three variables within the design space. Further experiments were conducted in which the spot tests that were not involved in the model design and that were located in the interior area of study (run 21, 22 23 and 24) (Table 2) were taken into account. Those four experimental combinations were used to study the biosurfactant production under the experimental conditions described above.

Bioassays

Bioassays were carried out using third instar *P. oleae* larvae of under starvation for 20 h, kindly provided by the Institute of Olive

Tree, Sfax, Tunisia. Fifty microliters of *B. subtilis* SPB1 biosurfactant suspension were poured on the surface of one olive leaf. The latter was left in Petri dish for 4 h then 10 larvae of *P. oleae* were introduced and incubated at room temperature in order to expose larvae to the diet containing the biosurfactant. 10 *P. oleae* larvae were fed with untreated olive leaf used as negative control. Five concentrations (50, 100, 200, 300 and 400 µg/ml) of the biosurfactant were tested in three replicates. Experiments were for 1 to 3 days followed by counting the number of dead larvae. 50% lethal concentration (LC₅₀) was calculated from pooled raw data by probit analysis using programs written in the R. language (Venables and Smith, 2004).

Preparation and sectioning of insect tissues

After exposure to *B. subtilis* SPB1 biosurfactant, *P. oleae* larvae were placed in 10% formol. Larvae were dehydrated in increasing ethanol concentrations, rinsed in 100% toluene and embedded in paraffin wax. Five-micrometer sections were obtained and placed in carriers loaded with a mix of 1.5% egg albumin and 3% glycerol in distilled water. For histopathological localization of the effects of *B. subtilis* SPB1 biosurfactant, the 5 µm sections already deparaffinated in 100% toluene were stained with hematoxylineosin (HE) as reported by Ruiz et al. (2004).

RESULTS

Insecticidal activity of *B. subtilis* SPB1 biosurfactant on *P. oleae*

When tested against third instars larvae of *P. oleae*, *B.*

Table 2. Estimate regression coefficients for biosurfactant production using data in coded units.

Nom	Estimate coefficient	F. Inflation	Standard deviation	t. experimental	Signification %
b ₀	4.253		0.0449	94.61	<0.01***
b ₁	0.070	1.00	0.029	2.36	6.5
b ₂	-0.171	1.00	0.029	-5.72	<0.01***
b ₃	-0.217	1.00	0.029	-7.27	<0.01***
b ₁₋₁	-1.229	1.02	0.029	-7.90	<0.01***
b ₂₋₂	-0.318	1.02	0.029	-10.95	<0.01***
b ₃₋₃	-0.318	1.02	0.029	-10.95	<0.01***
b ₁₋₂	0.515	1.00	0.038	13.21	<0.01***
b ₁₋₃	0.060	1.00	0.038	1.54	18.4
b ₂₋₃	0.040	1.00	0.038	1.03	35.2

(***): significant at the level 99.9%.

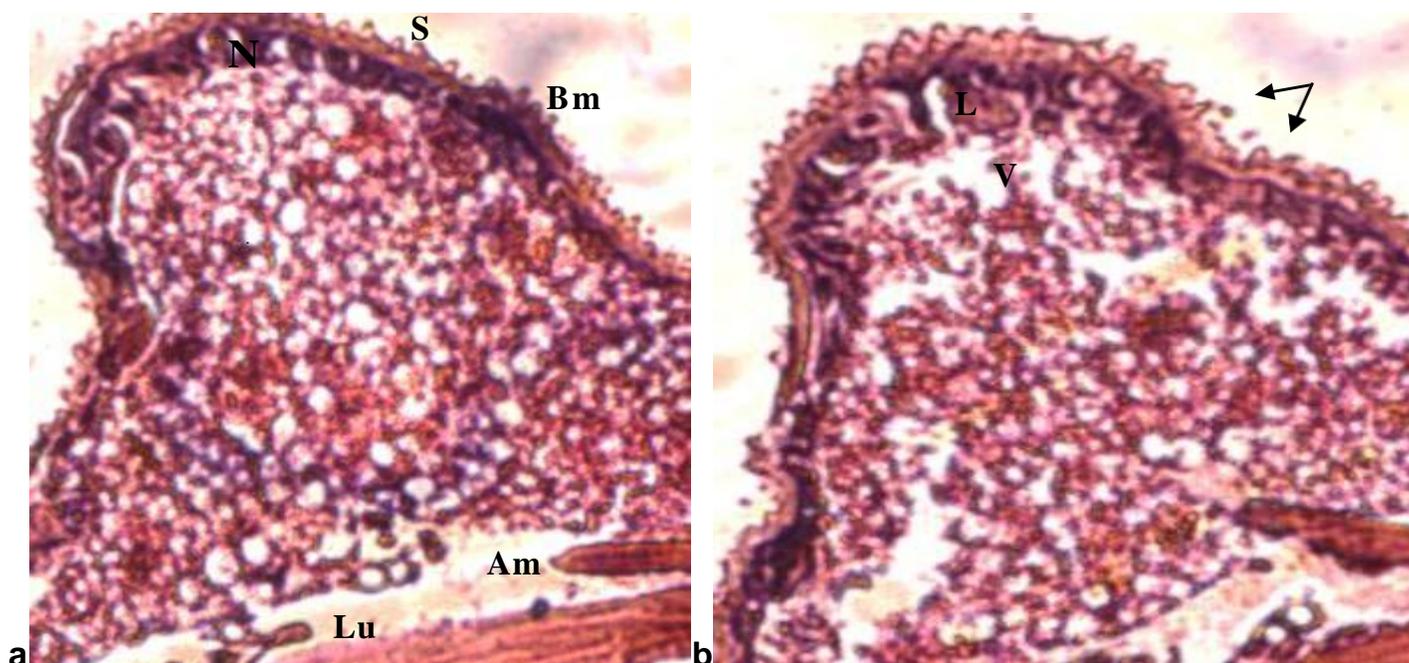


Figure 1. General aspects of the midgut of *P. oleae*; **a** and histopathological effects of *Bacillus subtilis* SPB1 biosurfactant on it; **b** Arrows indicate vesicle formation in the apical region of cells. L, lysis of columnar cells; Lu, lumen; N, nucleus; S, striated border; V, vacuole. a and b Magnification $\times 40$.

subtilis SPB1 biosurfactant showed toxicity with an LC₅₀ of 142 $\mu\text{g/ml}$ with 95% confidence limits of (102 to 182 $\mu\text{g/ml}$) and LC₉₀ of 369 $\mu\text{g/ml}$ with 95% confidence limits of (291 to 493 $\mu\text{g/ml}$). Moreover, the non-killed larvae exposed to SPB1 biosurfactant remained at the third-instar stage for more than 9 days. Exposure of larvae to untreated diet, used as negative control, did not cause mortality. These findings clearly demonstrate that the *B. subtilis* SPB1 biosurfactant is active against the olive moth *P. oleae*. The Lepidoptera larvicidal activity exhibited by this biosurfactant is novel because no such reports are available till date.

Histopathological effects of *B. subtilis* SPB1 biosurfactant in *P. oleae* larvae

Histopathological observations of the SPB1 biosurfactant effects on *P. oleae* were studied on third instar larvae which had been fed a diet containing the SPB1 bioemulsifier. As shown in Figure 1b, extensive damage is detected in the midgut epithelium, indicating that the midgut tissue is a primary site of action of the SPB1 biosurfactant. Mostly histopathological modifications included vacuolization of the cytoplasm, brush border membrane destruction, vesicle formation in the apical

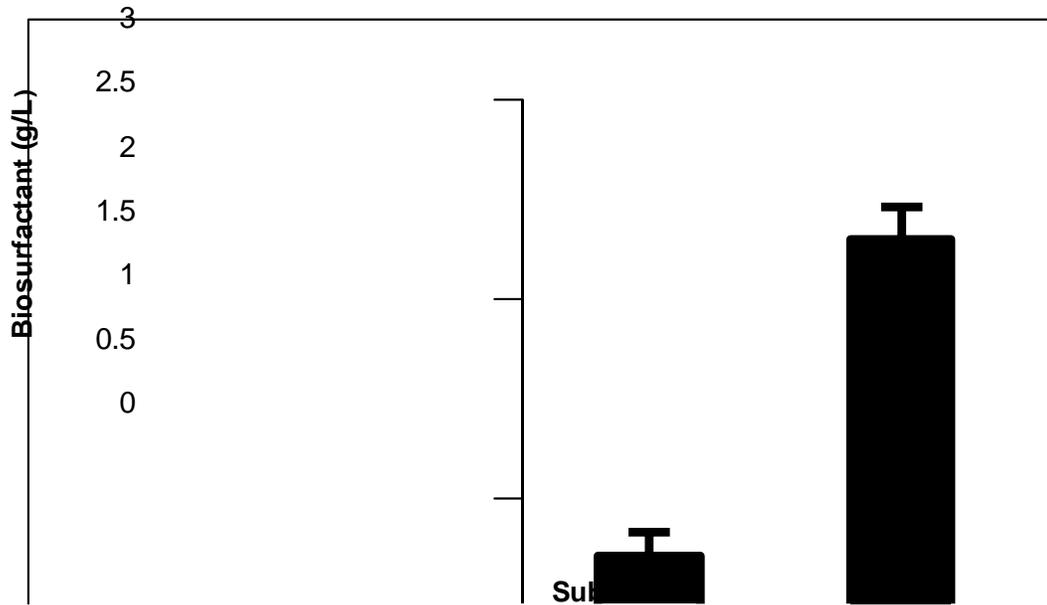


Figure 2. Evaluation of biosurfactant production by *B. subtilis* SPB1 strain using various substrates.

region of cells toward the midgut lumen and disintegration of the cells. In the control tissue, the midgut of untreated *P. oleae* larvae showed uniform morphology and well-defined epithelial cells with unaffected apical microvilli membrane (Figure 1a).

Effect of different substrates on biosurfactant production

The use of inexpensive substrates for the production of biosurfactant has combined benefit of utilizing a low-grade substrate while producing a commercially valuable product. The selection of a suitable substrate for the fermentation process is a critical factor (Tanyildizi et al., 2007) and thus involves the screening of a number of agro industrial materials for microbial growth and product formation. Different substrates are tested here, such as potato peels, chick-pea flour, wheat its, wheat flour, millet, soya bean meal, barley flour, orange peels, banana peels, barley its, rice flour and corn starch for the production of biosurfactant by submerged fermentation. As shown in Figure 2, maximum biosurfactant production (2.78 g/L) was obtained in a medium containing soya bean meal as the substrate. Similar production (2.63 g/L) could be obtained when using orange peels. Therefore, these tow later substrates were chosen for the formulation of biosurfactant production medium for *B. subtilis* SPB1 strain using response surface methodology.

Optimization of SPB1 biosurfactant production using response surface methodology

In order to formulate an economical medium adequate for

high biosurfactant production, various components of the medium and operational parameters were tested using Placket Burman screening design (data not shown). The effective variables that play a direct role in biosurfactant production were chosen for the further experiments using central composite design. Soya bean meals, orange peels and sea water were found to be good sources for the biosurfactant production. To study the individual and interactive effects of these variables and to optimize their levels, RSM was applied. The results are given in Table 1. The model was evaluated statistically using Nemrodw Version 2007 software (LPRAI, Marseille, France). It can be noted that there was a considerable variation in the amount of biosurfacatnt production yield and that this variation depended heavily on the levels of the three independent variables in the medium that varied from 2.39 g/L (run 7) to 4.32 g/L (run 1). By applying a least-squares method to the experimental data, the following second-order polynomial equation was found to adequately explain biosurfactant production by considering only the significant terms (Table 2).

$$Y = 4.253 - 0.171X_2 - 0.217X_3 - 1.229X_1^2 - 0.318X_2^2 - 0.318X_3^2 + 0.515X_1X_2$$

Where Y is the predicted response (biosurfactant production); X_1 , X_2 and X_3 are the coded values of orange peels, soya bean meal and sea water, respectively. The model equation was submitted to statistical analysis to settle on the coefficient R^2 which was calculated to be 0.932 for biosurfactant production showing that 93.2% of experimental data were compatible with the data predicted by the model. The predicted R^2 of 0.828 was in reasonable agreement with the adjusted R^2 of 0.872 indicating that regression model

Table 3. ANOVA analysis for response surface quadratic model.

Parameter	Sum of squares	df	Mean square	F-value	p-value Prob>F	Significance
Regression	6.3545	9	0.7061	58.1112	<0.001	Significant
Residual	0.4607	10	0.0461			
Lack of fit	0.4000	5	0.0800	6.5837	<0.001	Significant
Pure error	0.0608	5	0.0122			
Total	6.8152	19				

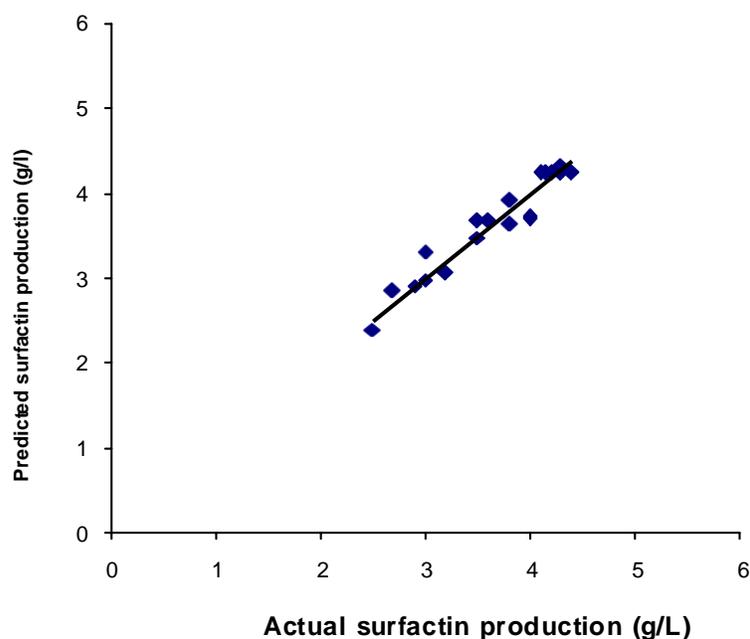


Figure 3. Parity plot showing the distribution of experimental versus model predicted values for *B. Subtilis* biosurfactant production.

could be used to analyze trends of responses. The closer the value of R (multiple correlation coefficients) to 1, the better the correlation between the observed and predicted values (Sayyad et al., 2007). The ANOVA analysis was done to investigate the effect of the various factors on the variation about the mean. Statistical testing of the model was done by the Fisher's statistical test and the results are shown in Table 3. The analysis of variance of the quadratic regression model demonstrates that the model is highly significant, as the computed F value is much greater than the tabular F value.

The student t distribution and the corresponding P values, along with the parameter estimate, are given in Table 2. The P values are used as a tool to check the significance of each of the coefficients which are, in turn, necessary to understand the pattern of the mutual interactions between the best variables. In fact, when the magnitude of the t test value is large and the P value is small, this indicates that the corresponding coefficient is

highly significant (Karthikeyan et al., 1996). As far as the current study is concerned, the estimated parameters and the corresponding P values suggest that all the independent and interactive terms, only b_{1-3} and b_{2-3} , had a significant effect on *B. subtilis* biosurfactant production (Tables 2 and 3). The parity plot (Figure 3) showed a satisfactory correlation between the experimental and the predicted values of biosurfactant production, wherein, the points cluster around the diagonal line indicated the optimal fit of the model, particularly because the deviation between the experimental and predicted values was minimal.

Response surface plots

The 3D response surface curves and their respective contour plots provided information about the interaction between two parameters and allowed an easy prediction

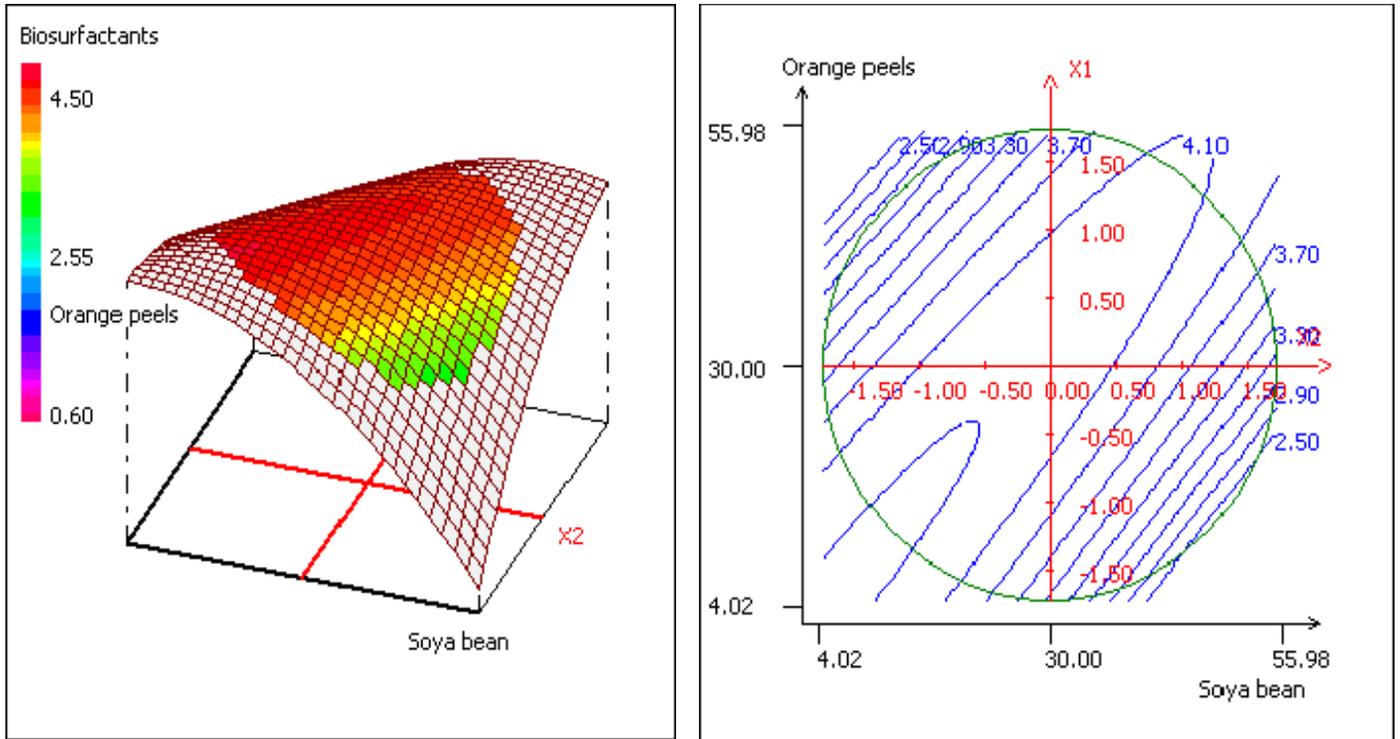


Figure 4. Effect of media component concentrations on the production of biosurfactant: response surface plot (left) and its contour plot (right) of interaction between orange peels and soya bean meals.

and interpretation of results. These plots are helpful in studying the effects of the factors within the experimental space and consequently, in determining the optimal factors levels (Kammoun et al., 2008). The interaction of three medium components used for biosurfactant production by *B. subtilis* was investigated by plotting the 3D response surfaces with the vertical axis representing biosurfactant production and two horizontal axes representing the coded levels of two explanatory factors. In each plot, the factor, which not representing the two horizontal axes, was fixed at its central actual level. The response surface plots and corresponding contour plots are given in Figures 4 and 5. Figure 4 shows the effects of orange peels and soya bean meals on biosurfactant production when sea water percentage was fixed at its middle level (30%). It can be noted that at a low orange peels concentration, the increase in soya bean concentration induced a significant decrease in biosurfactant production from 4.3 g/L to 2.5 g/L.

This finding suggests that biosurfactant production was better induced at low orange peels and soya bean concentrations. By further subjecting the data from Figure 4 to a Nemrod-W software analysis, it was found that the maximum predicted value of biosurfactant production was 4.3 g/L. In this case, the optimum orange peels and soya bean concentrations and in the uncoded units were 15.5 and 10 g/L, respectively. Figure 5 shows the effect of soya bean concentration and sea water percentage on

biosurfactant production when orange peels concentration was fixed at its middle level (30 g/L). The contour plots are co centric and parallels to each other. The maximum of biosurfactant production is globally located in the central experimental space with soya meal concentration and sea water percentage lower than central values. However a decrease was observed in biosurfactant production elsewhere this region especially at high and low sea water percentages. The maximum predicted value for biosurfactant production obtained was 4.3 g/L. In this case, the optimum soya bean concentration and sea water percentage in the uncoded units were 22 g/L and 26%, respectively.

Optimum validation

The response surfaces allowed the determination of the optimal levels of variables for maximum biosurfactant production yield. The value of X_1 (orange peels flour concentration), X_2 (soya bean flour concentration), and X_3 (sea water dilution) were found to be 15.5, 10 g/L and 30%, respectively. The corresponding experiment was carried out in five replicates and the average yield value was calculated. The biosurfactant production was 4.45 g/L while the predicted value was 4.3 (± 0.21) g/L. This production was, interestingly two fold much higher than that obtained when using the defined medium composed

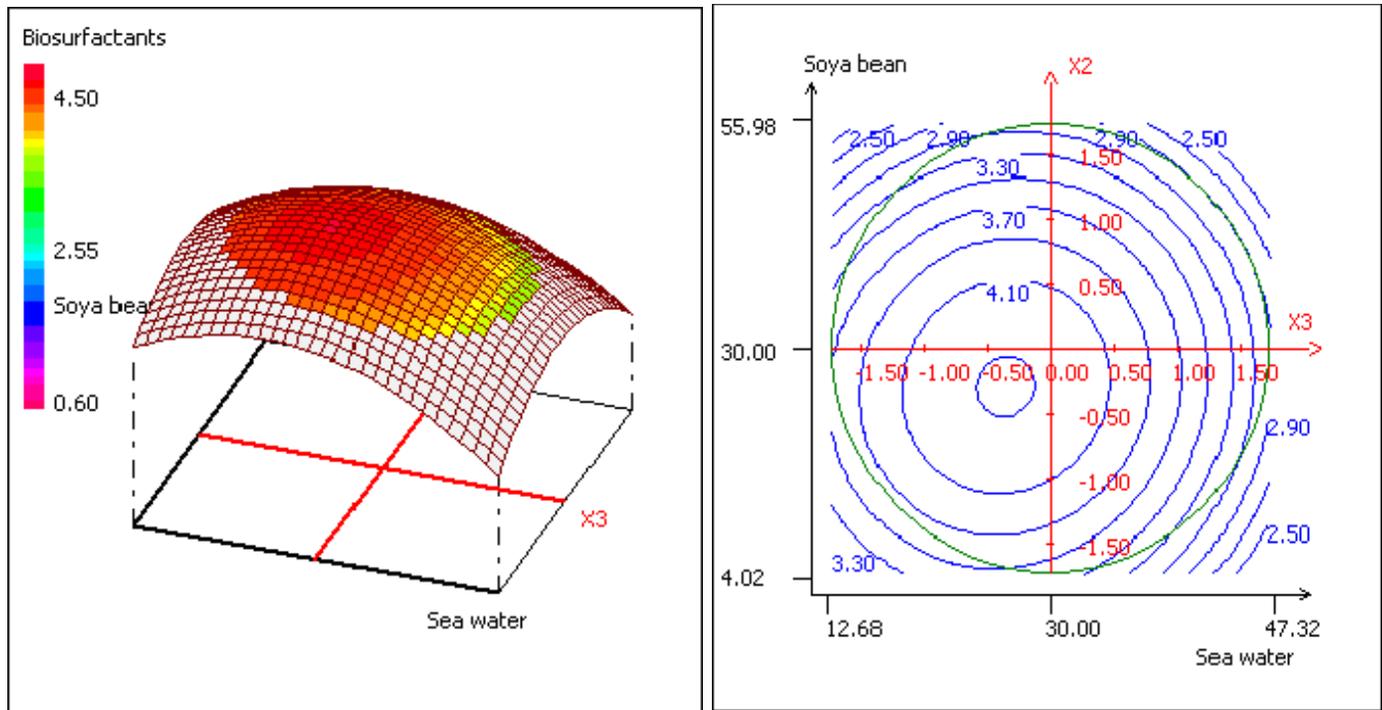


Figure 5. Effect of media component concentrations on the production of biosurfactant: response surface plot (left) and its contour plot (right) of interaction between soya bean meals and sea water.

Table 4. Data for the validation of the experimental model.

Run order	Variable values ^a			Biosurfactant production (g/L)	
	X1 (g/L)	X2 (g/L)	X3 (%)	Experimental	Predicted
21	19.39	23.88	27.11	4.40	4.42
22	40.61	23.88	27.11	3.98	4.08
23	30	42.25	27.11	3.42	3.68
24	30	30	38.66	4.00	3.84

^a X1 Orange peels; X2 Soya bean; X3 Seawater; $R^2 = 0.91$.

of basal salts, urea and glucose.

Validation of the model

The model was verified for the three variables within the design space. Four combinations of production conditions were selected by the software and were then tested for biosurfactant production (Table 4). The experimental values of biosurfactant production that were determined were found to be in good agreement with those that were statistically predicted by the model ($R^2=0.91$), thus confirming the authenticity and reliability of the model. In addition, the average error (difference between observed and predicted value) was close to zero, indicating the absence of bias in the predictions made by the model.

DISCUSSION

The olive moth, *P. oleae*, is one of the most important insect pests to olives in the Mediterranean basin and from Mexico to Southern America. This insect reduces the overall yields (10%) and quality of the fruit/oil. It could be controlled efficiently only by chemical pesticides, causing toxicity to consumers and other beneficial insects and increasing the costs of production. Thus, there was increasing demand for alternative methods of controlling this insect. Microbial bioinsecticides were shown to be an efficient tool for the control of plant pests. The Gram-positive, spore-forming bacterium *B. subtilis* produces several lipopeptides biosurfactants exhibiting insecticidal activity especially against diptera. In this work, we showed for the first time the laticidal potency of *B.*

subtilis SPB1 biosurfactant against lepidoptera larvae. Indeed, this bioemulsifier attacks the midgut of *P. olea* larvae causing disruption of epithelial cells and leakage of material in the lumen (Figure 1). Similar vesicles formation in the apical region was previously described in *Ephestia kuehniella* (Smith et al., 1996) and *P. oleae* (Rouis et al., 2007) larvae treated with *Bacillus thuringiensis* Cry toxins and in *Agrotis ipsilon* larvae fed with Vip3A-containing diet (Yu et al., 1997).

This demonstrated that *B. thuringiensis* Cry and *B. subtilis* biosurfactant have similar histopathological effects on susceptible lepidopteran larvae. The degeneration of midgut cells conducts to larval death. In fact, the SPB1 biosurfactant causes *P. olea* mortality with an LC₅₀ and LC₉₀ of 142 and 369 µg/ml, respectively. The present study showed, for the first time, that *B. subtilis* SPB1 biosurfactant is an efficient biological agent against *P. olea*. Efforts must therefore be redirected to improve production efficiency of this compound and to reduce its cost. The use of statistical models to optimize culture medium components and conditions has enhanced in the present-day biotechnology research, due to its ready applicability and aptness. Many researchers have attempted to produce biosurfactants by using inexpensive carbon and nitrogen sources in culture medium (Mercede and Manera, 1994). Selection of waste substrates involves the problem of finding a waste with the right balance of carbohydrates and lipids to support optimal growth and production (Makkar and Cameotra, 2002). Agroindustrial wastes, with high levels of carbohydrates or lipids and urban wastes meet the requirements for use as substrates for biosurfactant production (Makkar and Cameotra, 2002). Peat pressate, urban waste, olive oil mill effluent and lactic whey are possible substrates for surfactant accumulation.

Apart from traditional carbon and nitrogenous substrates, the spectrum of available raw materials includes various agricultural and industrial by-products and waste materials. These agricultural feed stocks are attractive in that they are available in surplus and can be produced in regions with temperate to tropical climates. Potential substrates for surfactant production have been sought that might provide cheaper and renewable sources for economical production (Maneerat, 2005). Interesting cheap sources have been described from agroindustrial crops and residues, such as cassava (Nitschke and Pastore, 2006), soybean, sugar beet, potato (Noah et al., 2005) and sweet sorghum, from crop residues such as bran and straw of wheat and rice (Ohno et al., 1992) and from waste derived from oil processing mills, for example soy molasses from soybean processing and the crude glycerol fraction from biodiesel production (Solaiman et al., 2005).

There have been no detailed reports concerning utilisation of orange peels for the production of *B. subtilis* biosurfactants. Moreover, sea water has also been reported to be a good source of mineral salts (NaCl,

MgSO₄, MnSO₄, FeSO₄...) that would substitute perfectly the individual supply of all the minerals into the culture medium used for *B. thuringiensis* bioinsecticides production (Ghribi et al., 2007). The central composite design exploited in the present study enabled us to investigate the culture medium composition that support twofold increase in *B. subtilis* SPB1 biosurfactant production compared to that obtained when using the defined medium composed of basal salts, urea and glucose (Ghribi and Ellouze-Chaabouni, 2011). By increasing biosurfactant yield via the experimental design approach, the production cost of the biosurfactant would markedly be reduced, enhancing feasibility of commercial application of this powerful biosurfactant. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of response surface methodology to optimize the process for biosurfactant production. A maximum biosurfactant production of 4.45 g/L was achieved when using medium composed only of 15.5 g/L orange peels and 10 g/L soya bean mixed with 30% of diluted sea water. Validation experiments were also carried out to verify the adequacy and the accuracy of the model and results showed that the predicted value agreed with the experimental values well and more than 2 fold increase compared to the original medium was obtained.

The results also give a basis for further study with large scale fermentation for *B. subtilis* biosurfactant production. The most reported studies dealing with the optimization of medium components using statistical approaches for low-cost *B. subtilis* biosurfactants production concerned the use of solid state fermentation process (Wei et al., 2007; Muthusamy et al., 2008) and no studies reported *B. subtilis* biosurfactant production under submerged fermentation. In the present study, the complex medium is only composed of orange peels and commercial quality soya bean meal mixed in 30 % diluted seawater. The medium consists of cheap components available on a commercial "local" scale, thereby not only making it industrially relevant but also improving the toxicity against *P. olea* larvae. Further, the formulated medium could be efficiently used at large scale fermentation for *B. subtilis* biosurfactant low cost production in biotechnology industries.

REFERENCES

- Association of Official Analytical Chemistry - AOAC. (1984), Official methods of analysis of the Association of Official Analytical Chemists. 14. ed. Arlington, pp: 1141.
- Banat IM, Franzetti A, Gandolfi, Bestetti G, Martinotti MG, Fracchia L, Smith TJ, Marchant R (2010). Microbial biosurfactants production, applications and future potential. Appl. Microbiol. Biotechnol., 87: 427-444.
- Desai JD, Banat IM (1997). Microbial production of surfactants and their commercial potential. Microbiol. Molec. Biol. Rev., 61: 47-64.
- Dubois M, Gilles K, Hamilton J, Rebers P, Smith F (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350-356.

- Eeman M, Berquand A, Dufrene YF, Paquot M, Dufour S, Deleu M (2006). Penetration of surfactin into phospholipid monolayers: nanoscale interfacial organization. *Langmuir*, 22(26): 11337-11345.
- Griffin HL, Greene RV, Cotta MA (1992). Isolation and characterization of an alkaline protease from the marine shipworm bacterium. *Curr. Microbiol.*, 24: 111-117.
- Ghribi D, Zouari N, Trigui W, Jaoua S (2007). Use of sea water as salts source in starch and soya bean based media for the production of *Bacillus thuringiensis* bioinsecticides. *Process Biochem.*, 42: 374-378.
- Ghribi D, Elouze-Chaabouni S (2011). Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol. Res. Int.* doi:10.4061/2011/653654.
- Heerklotz H, Seelig J (2007). Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *Europ. Biophys. J.*, 36: 305-314.
- Karthikeyan RS, Rakshit SK, Baradarajan A (1996). Optimization of batch fermentation conditions for dextran production. *Bioprocess Eng.*, 15: 247-251.
- Lu JR, Zhao XB, Yaseen M (2007). Biomimetic amphiphiles: Biosurfactants. *Curr. Opin. Interf. Sci.*, 12: 60-67.
- Kammoun R, Naili B, Bejar S (2008). Application of a statistical design to the optimization of parameters and culture medium for α -amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product). *Biores. Technol.*, 99(13): 5602-5609.
- Mercede ME, Manersa MA (1994). The Use of Agroindustrial By-products for Biosurfactant Production. *J. Am. Oil Chem. Soci.*, 71: 61-64.
- Makkar RS, Cameotra SS (2002). An update on the use of unconventional substrates for biosurfactant production and their new applications. *Appl. Microbiol. Biotechnol.*, 58: 428-434.
- Maneerat S (2005). Production of biosurfactants using substrates from renewable-resources. *Songklanakarin J. Sci. Technol.*, 27: 675-683.
- Mukherjee S, Das P, Sen R (2006). Towards commercial production of microbial surfactants. *Trend. Biotechnol.*, 24: 509-515.
- Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008). Biosurfactants: Properties, commercial production and application. *Curr. Sci.*, 94: 736-747.
- Nair SR, Panda T (1997). Statistical optimization of medium components for improved synthesis of pectinase by *Aspergillus niger*. *Bioprocess Eng.*, 16: 169-173.
- Noah KS, Bruhn DF, Bala GA (2005). Surfactin Production from Potato Process Effluent by *Bacillus subtilis* in a Chemostat. *Appl. Biochem. Biotechnol.*, 122(1-3): 465-474.
- Nitschke M, Pastore GM (2006). Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. *Biores. Technol.*, 97(2): 336-34.
- Ohno A, Takashi A, Shoda M (1992). Production of a Lipopeptide Antibiotic Surfactin by Recombinant *Bacillus subtilis* NB22 Using Wheat Bran as Substrate. *Biotechnol. Lett.*, 14: 817-821.
- Pearson D (1970). *The Chemical Analysis of Foods* (7th edition). Churchill Livingstone, Edinburgh, pp. 6-25.
- Rahman KSM, Thahira JR, Stephen MC, Roger M, Ibrahim MB (2002). Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw material. *Biotechnol. Prog.*, 18: 1277-1281.
- Ruiz LM, Segura C, Trujillo J and Orduz S (2004). *In vivo* binding of the Cry11Bb toxin of *Bacillus thuringiensis* subsp. *Medellin* to the midgut of mosquito larvae (Diptera: Culicidae). *Memórias do Instituto Oswaldo Cruz.*, 99: 73-79.
- Rouis S, Chakroun M, Saadaoui I, Jaoua S (2007). Proteolysis, histopathological effects, and immunohistopathological localization of d-endotoxins of *Bacillus thuringiensis* subsp. *Kurstaki* in the midgut of Lepidopteran olive tree pathogenic insect *Prays oleae*. *Molec. Biotechnol.*, 35: 141-148.
- Smith DS, Compher K, Janners M, Lipton C, Wittle LW (1969). Cellular organization and ferritin uptake in the midgut epithelium of the moth *Ephesia kuehniella*. *J. Morphol.*, 127: 41-72.
- Solaiman D, Ashby RD, Foglia TA (2005). Production of biosurfactants by fermentation of fats, oils and their coproducts. *In Handbook of Industrial Biocatalysis*. Edited by: Hou CT. Boca Raton, FL: Taylor & Francis., pp. 1-9.
- Sayyad SA, Panda BP, Javed S, Ali M (2007). Optimization of nutrient parameters for lovastatin production by *Monascus purpureus* MTCC 369 under submerged fermentation using response surface methodology. *Appl. Microbiol. Biotechnol.*, 73: 1054-1058
- Tanyildizi MS, Özer D, Elbol M (2007). Production of bacterial α -amylase by *B. amyloliquefaciens* under solid substrate fermentation. *Biochem. Eng. J.*, 37: 294-297.
- Venables WN, Smith DM (2004). The R. development core team. An introduction to R. Notes on R: A Programming Environment for Data Analysis and Graphics Version 1.9.1.
- Wei YH, Lai CC, Chang JS (2007). Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. *Process Biochem.*, 42: 40-45.
- Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ (1997). The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl. Env. Microbiol.*, 63: 532-536.