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International Journal of Pharmacy and Biological Sciences (eISSN: 2230-7605)
IJPBS |Volume 1| Issue 3 |JULY- SEPT |2011|103-116

OPTIMIZATION OF FERMENTATIVE PRODUCTION OF SOPHOROLIPID BIOSURFACTANT BY *STARMERELLA BOMBICOLA NRRL* Y-17069 USING RESPONSE SURFACE METHODOLOGY

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Research Article

RECEIVED ON 01-07-2011 **ACCEPTED ON 20-07-2011**

ABSTRACT

Sophorolipids (SLs) are glycolipids type of biosurfactants and are produced by few of the non-pathogenic yeast species like Starmerella bombicola. In the present work, statistical experimental methodology was used to optimize the fermentative production of SLs from Starmerella Bombicola NRRL Y-17069 at the shake flask scale. The Placket–Burman screening experiments was applied to evaluate the significant variables that influence the production of Sophorolipids. It was found that pH, concentration of Yeast extract and the Concentration of Oleic acid are the most influential variables that affected the production of Sophorolipids. The optimum levels of these three variables were achieved by using a Box-Behnken design of the response surface methodology (RSM). The predicted maximal sophorolipid production of 18.32 g/L appeared at pH 3, and when the concentrations of yeast extract and oleic acid were 5 g/L, and 20 g/L, respectively. Under the proposed optimized conditions, the sophorolipid production reached 18.2 g/L. The correlation between predicted value and measured value of these experiments proved the validity of the response model.

KEYWORDS: Fermentation, Sophorolipids, Biosurfactants, *Starmerella bombicola,* Response surface methodology (RSM).

INTRODUCTION

Biosurfactants made by fermentation from renewable resources provide "environmental friendly" processes and products. Sophorolipids are surface-active glyco-lipid compounds synthesized by few of the non-pathogenic yeast species like *Candida bombicola* (Starmerella bombicola)[1,2], *Wickerhamiella domericqiae*[3], *Rhodotorula bogoriensis* [4] etc. Apart from their surface active properties, Sophorolipids are also found to possess antimicrobial, anticancer and, spermicidal activity [5]. Further they are also a source of difficult-to synthesize ω and ω-1 hydroxy fatty acids, which find application in the perfume and fragrance industry [5]. Plackett-Burman and Box-Behnken designs are among the most widely used statistical techniques for optimization of biological processes. The Plackett-Burman experimental design is a two-level factorial design, which identifies the

critical physicochemical parameters by screening *N* variables in *N*+1 experiments [6], but it does not consider the interaction effect among the variables. The variables that are found significant in this initial screening can be further optimized using response surface methodology (RSM). Response surface methodology (RSM) has been used extensively in media optimization. RSM is a collection of statistical techniques that uses design of experiments (DoE) for building models, evaluating the effects of factors and predicting optimum conditions [7-10].

To the best of our knowledge, there are no reports on the application of statistical methods for the optimization of sophorolipid production in submerged fermentation at shake flask scale. Here, we have made an attempt to optimize production of sophorolipid by using *Starmerella bombicola* through a two stage optimization process. In stage one the "Placket–Burman" screening experiments were applied to evaluate the significant variables that influence the production of sophorolipids. In stage two "Box-Behnken design" of response surface methodology (RSM) was used to evaluate the effects of factors and predicting optimum conditions.

MATERIALS AND METHODS:

Microorganism

The yeast *Starmerella bombicola NRRL Y-17069*, capable of producing large amounts of sophorolipids was obtained from ARS Culture Collection, USA. The organism was maintained at 4 °C on Potato Dextrose Agar (PDA) slants and was sub-cultured monthly.

Medium composition and culture conditions

The basal medium used for sophorolipid production contained Glucose 100 g/L, Oleic acid 100 g/L, yeast extract 10 g/L and urea 1 g/L. The 250-ml Erlenmeyer flasks containing 50 ml of the medium were inoculated with 2 ml of 48 hour grown inoculum and were incubated in a rotary shaker for 10 days at 30 °C and 180 rpm.

Preparation of the pre-culture and inoculum

The pre-culture medium contained 100 g/L of glucose, 10 g/L of yeast extract and 1 g/L of urea. The 250-ml Erlenmeyer flasks containing 50 ml of the medium were inoculated with 2 ml of organism (prepared by adding 10 ml of saline to PDA slant culture) and were incubated in a rotary shaker for 48 hr at 30 °C and 180 rpm to produce the inoculum.

Preparation and inoculation of the production media

Production media with different media composition were made as stated in **Table 1** and **Table 2** for screening of the significant variables and optimization of significant variables respectively. Required pH was adjusted using 0.1M citrate buffer. Two ml of the Inoculum was added to the 50ml of the production media and were incubated in a rotary shaker at 30ºC and 180 rpm. Oleic acid was autoclaved separately and was added aseptically after 48th hour of inoculation of the production medium and fermentation was allowed to take place further for a total period of 240 h.

Optimization of sophorolipid production

The optimization of physicochemical factors for Sophorolipid production was carried out in two stages.

Stage 1: Screening of physicochemical factors using plackett-burman design

Plackett-Burman experimental design consisting of a set of 8 experiments was used to determine the relative significance of 7

factors that influenced sophorolipid production by *S.bombicola* in submerged fermentation at shake flask scale. The complete experimental design is shown in **Table 1**. The software Design of Experiments (DOE++, Trial Version 1.0.6, ReliaSoft Corporation, USA) was used for experimental design. The factors or independent variables considered for study included one physical factor (pH), and six nutritional factors (concentrations in g/L of glucose, yeast extract, urea, FeSO4, Oleic acid and NaCl). All variables were numerical factors and were investigated at two widely spaced levels designated as –1 (low level) and +1 (high level). All trials were performed in triplicate and the average of the sophorolipid yield and yield coefficient (defined as grams of sophorolipid produced per 100 g of the carbon source) were used as responses R1 and R2 respectively.

The main effects for each of these factors were defined and calculated by Eq. 1.

$E_I=(R^*_+)_I-(R^*_-)_I$ … (Eq. 1)

Where E_I is the effect of the I^{th} factor on the response and $(R^*_+)_I$ and $(R^*_-)_I$ are the average response values at the high (+) and low (-) levels of the factor [11, 12].

Stage 2: Optimization of significant variables using box-behnken design

Response surface methodology using Box-Behnken design was used to determine the optimum levels of the significant variables (pH, Yeast extract, Oleic acid) and the effects of their mutual interactions on sophorolipid production. A total of 15 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as –1, 0 and +1, respectively). The center point of the design was replicated three times for the estimation of error. The experimental design used for the study is shown in **Table 2**. The software Design-Expert (Trial Version 8.0.1.0, Stat-Ease, Inc,USA) was used for experimental design, and data analysis. A multiple regression analysis of the data was carried out to define the response in terms of the independent variables. The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum sophorolipid production. All trials were performed in triplicate and the average of the sophorolipid yield and yield coefficient were used as responses R1 and R2 respectively.

Isolation of sophorolipids

Spent cultures medium was centrifuged at 5000×g for 10 min. The sediment containing mixture of cell mass and the produced sophorolipids was extracted with 50 ml of ethyl acetate in a 250 ml Erlenmeyer's flask and by shaking in a rotary shaker at 180 rpm for 30 min. The extract was again centrifuged at 1500×g for 2 min for separating the cell mass and the extract. The solvent was removed from the extract by rotary evaporation. The Amber colored, honey like semi-crystalline product (sophorolipids) was washed twice with 15ml of n-Hexane to remove the unused oleic acid, and was stored at 4ºC.

Table 1 : Plackett – Burman experimental design

Trial	Factors							R1*	R2*
	Glucose	Yeast extract	Urea	pH	Oleic acid	$FeSO_4$	NaCl		
1	1	1	1	-1	1	-1	-1	6.84	3.42
2	-1	1	1	1	-1	1	-1	3.58	4.45
3	-1	-1	1	1	1	-1	1	0.5	0.36
4	1	-1	-1	1	1	1	-1	0.8	0.4
5	-1	1	-1	-1	1	1	1	5.8	4.14
6	1	-1	1	-1	-1	1	1	4.36	3.11
7	1	1	-1	1	-1	-1	1	3.56	2.54
8	-1	-1	-1	-1	-1	-1	-1	2.7	3.375

Factors	**Low level (-1)**	**High level (+1)**
Glucose	40 g/L	100 g/L
Yeast extract	1 g/L	10 g/L
Urea	0 g/L	1 g/L
pH	4	6
Oleic acid	40 g/L	100 g/L
$FeSO_4$	0 g/L	0.2 g/L
NaCl	0 g/L	0.2 g/L

+1: high level; –1: low level;
R1 (Response1): sophorolipid yield (g/L);
R2 (Response2): Yield coefficient [Grams of sophorolipids per 100 grams of carbon source (glucose + oleic acid)]
* Values indicate mean of triplicate observations.

Table 2 : Box-Behnken design matrix with experimental and predicted values of production of sophorolipids by *S. bombicola*

	Factors (coded values)[a]			R1		R2	
Trial	**pH**	**Yeast extract**	**Oleic acid**	**Experimental***	**Predicted**	**Experimental***	**Predicted**
1	-1	-1	0	21.32	20.19	26.65	25.61
2	+1	-1	0	1.53	1.91	1.91	2.44
3	-1	+1	0	16.24	15.85	20.3	19.76
4	+1	+1	0	6.72	7.84	8.4	*9.43*
5	-1	0	-1	15.14	15.23	25.23	25.30
6	+1	0	-1	4.51	3.09	7.51	6.01
7	-1	0	+1	15.43	16.84	15.43	16.92
8	+1	0	+1	2.77	2.67	2.77	2.69
9	0	-1	-1	10.24	11.29	17.06	18.01
10	0	+1	-1	8.82	9.11	14.7	15.16
11	0	-1	+1	9.2	8.91	9.2	8.74
12	0	+1	+1	13.69	12.66	13.69	12.73
13	0	0	0	14.59	13.59	18.23	16.98
14	0	0	0	12.56	13.59	15.7	16.98
15	0	0	0	13.63	13.59	17.03	16.98

+1: high level; –1: low level; 0: medium level;
R1 (Response1): sophorolipid yield (g/L);
R2 (Response2): Yield coefficient (Grams of sophorolipids per 100 grams of carbon source)
***** Values indicate mean of triplicate observations.
[a] Real values (in sequence of -1, 0, +1) pH 3,4,5 ; yeast extract 5, 10,15 g/L, oleic acid 20, 40,60 g/L.

Table 3 Statistical calculations for Plackett–Burman design

Factor (variable)	$E_{sophorolipid\ yield}$	$E_{Yield\ coefficient}$
Glucose (g/L)	0.373	-0.357
Yeast extract (g/L)	1.32	1.018
Urea (g/L)	0.303	0.111
pH	-1.41	-0.787
Oleic acid (g/L)	0.123	-0.644
$FesO_4$ (g/L)	0.118	0.301
NaCl (g/L)	0.038	-0.187

RESULTS:

Screening of parameters using plackett-burman design

S. bombicola produced 3.2g of sophorolipids per 100g of oleic acid in the basal medium. The Plackett-Burman experimental design used for the screening of physicochemical factors influencing sophorolipid production by *S. bombicola* along with the corresponding experimental values of response were shown in **Table 1 and Table 3** shows the "E" value for each variable (indicative of its effect). The magnitude of the "E" value of the tested variable is indicative of its effect or its significance in altering the response, while the positive and the negative sign of the "E" value of a tested variable indicates its positive and negative influence on the responses respectively. Thus, the variables pH (having $E_{sophorolipid\ yield}$ of -1.41 and $E_{Yield\ coefficient}$ of -0.787) and Yeast extract concentration (having $E_{sophorolipid\ yield}$ of +1.32 and $E_{Yield\ coefficient}$ of +1.018) are the most significant variables since they have the highest "E" values for both the responses, indicating their strong influence on both overall sophorolipid yield and yield coefficient. Oleic acid concentration having $E_{sophorolipid\ yield}$ of + 0.123 and $E_{Yield\ coefficient}$ of -0.644 indicates that it does not contribute any significant influence on the sophorolipid yield; however it strongly influences the yield coefficient. All the other variables have comparatively lower "E" value (Table 3.) indicating their comparatively insignificant influence on both sophorolipid yield and yield coefficient, and thus their concentrations were kept constant at their coded value of -1. (Glucose at 40 g/L, Urea, $FeSO_4$ and Nacl at 0 g/L) in the subsequent experiments of optimization by RSM technique, while the concentration of yeast extract, pH and concentration of the oleic acid were considered for further optimization by RSM technique.

Optimization of significant variables using box-behnken design

The Box-Behnken design along with the corresponding experimental and predicted values of the sophorolipids yield is given in **Table 2**. The data were analyzed by multiple regression analysis using the Design-Expert software and after the regression analysis, following response models were obtained.

Sophorolipid yield = +13.59 -6.57(pH) +0.40(A) +0.30(B) +2.57(pH) (A) -0.51(pH)(B) +1.48(A)(B) $-1.58(pH)^2$ - $0.56(A)^2$ $-2.55(B)^2$ …(Eq 2.)

Yield Coefficient = +16.99 -8.38(pH) +0.28(A) -2.93(B) +3.2(pH) (A) +1.27(pH)(B) +1.71(A)(B) - 1.80($pH)^2$ $-0.87(A)^2$ $-2.45(B)^2$ … (Eq 3.)

Where:
A: Concentration of yeast extract (g/L).
B: Concentration of oleic acid (g/L)

The ANOVA of the quadratic regression model for the sophorolipid yield indicated that the model was highly significant, as the F-value for the model was 20.65. The Prob>F value of the model was 19×10^{-4} which indicates that there was only 0.19 % chance that the 'model F-value' this large could occur due to noise, which also confirmed that the model was highly significant. The estimated coefficient and the corresponding Prob>F values (**Table 4a**.) suggested that among the independent variables pH, multiple terms of pH and yeast extract and squared term of oleic acid had a significant effect on yield of sophorolipid. The model fitting values, which indicate model adequacy, are given in Table 5. The coefficient of variation (C.V. %), indicative of the degree of precision with which the treatments are compared, had a lower value (13.48 %), showing greater reliability. Also, the multiple regression coefficient (R^2) had a value of 0.974, indicating that the model could explain up to 97.4% of the variability of the response. The value of R^2 (0.974) also indicates good agreement between the experimental and predicted values of response. The signal to noise ratio (adequate precision) for the model was higher than 4 (14.981), indicating a good fit.

Similarly, the ANOVA of the quadratic regression model for the yield coefficient indicated that the model was highly significant, as the F-value for the model was 31.84. The Prob>F value of the model was 7×10^{-4}, which indicates that there was only 0.07 % chance that the 'model F-value' this large could occur due to noise, which also confirmed that the model was highly significant. The estimated coefficient and the corresponding Prob>F values (**Table 4b.)** suggested that among the independent variables pH, Oleic acid, multiple terms of pH and yeast extract and squared term of oleic acid had a significant effect on the yield coefficient. The model fitting values, which indicate model adequacy, are given in **Table 5**. The coefficient of variation (CV), indicative of the degree of precision with which the treatments are compared, had a lower value (11.14 %), showing greater reliability. Also, the multiple regression coefficient (R^2) had a value of 0.983, indicating that the model could explain up to 98.3 % of the variability of the response. The value of R^2 (0.983) also indicates a good agreement between the experimental and predicted values of response. The signal to noise ratio (adequate precision) for the model was higher than 4 (17.869), indicating a good fit.

The effect of the interaction of various physicochemical parameters on the sophorolipid production by S. bombicola was investigated by plotting the response surface curves against any two independent variables while keeping the third independent variable at the center (coded value of 0) level. Thus, three response surfaces for each response were obtained by considering all the possible combinations. The interactive roles of pH, Concentration of yeast extract and concentration of oleic acid on sophorolipid yield are illustrated in **Fig. 1** and interactive roles of pH, Concentration of yeast extract and concentration of oleic acid on yield coefficient are illustrated in **Fig. 2**. It is observed that the response surface curves for both sophorolipid yield as well as yield coefficient are identical except for the response surface of pH with oleic acid (**Fig. 1b and Fig. 2b**). It can be observed that the sophorolipid yield increases with decrease in the pH, at any given concentration of oleic acid, and at the lower pH there is a slight increase in the sophorolipid yield with an increase in the concentration of oleic acid (**Fig. 1b**). On other hand the yield coefficient also increases with decrease in the

pH, at any given concentration of oleic acid, however at the lower pH there is a slight decrease in the yield coefficient with an increase in the concentration of oleic acid (**Fig. 2b**). An increase in both sophorolipid yield as well as yield coefficient was observed when the pH was decreased together with a decrease in the concentration of yeast extract (**Fig. 1a and Fig. 2a**). The response surfaces in Fig. 1c and Fig. 2c shows the interactive effects of concentration of yeast extract and oleic acid on sophorolipid yield and the yield coefficient respectively.

Based on the above results, the software was also used to predict the optimum values of the three significant variables to optimize both sophorolipid yield as well its yield coefficient. It was found that the predicted optimum values of all the three variables were at the coded level of -1 (pH at 3, Concentration of yeast extract at 5 g/L and Concentration of the oleic acid at 20g/L) and the experimental values of the sophorolipid yield (18.20 g/L equivalent to a yield coefficient of 30.33) were only marginally lower than the predicted yield (18.32 g/L equivalent to a yield coefficient of 30.53) at the predicted optimum conditions.

Table 4a : Analysis of variance(ANOVA) of the response surface quadratic model for the sophorolipid yield

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	415.320	9	46.146	20.651	19×10^{-4}	significant
A-pH	345.845	1	345.845	154.774	$< 1\times10^{-4}$	
B-Yeast extract	1.264	1	1.264	0.565	0.485	
C-Oleic acid	0.708	1	0.708	0.316	0.598	
AB	26.368	1	26.368	11.800	0.018	
AC	1.030	1	1.030	0.461	0.527	
BC	8.732	1	8.732	3.907	0.105	
A^2	9.251	1	9.251	4.140	0.097	
B^2	1.150	1	1.150	0.514	0.505	
C^2	23.970	1	23.970	10.727	0.022	
Residual	11.172	5	2.234			
Lack of Fit	9.110	3	3.036	2.944	0.263	not significant
Pure Error	2.062	2	1.031			

Table 4b: Analysis of variance (ANOVA) of the quadratic regression model for the yield Coefficient

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	723.001	9	80.333	31.839	7×10^{-4}	significant
A-pH	561.460	1	561.460	222.530	$< 1\times10^{-4}$	
B-Yeast extract	0.644	1	0.644	0.255	0.635	
C-Oleic acid	68.503	1	68.503	27.150	0.003	
AB	41.216	1	41.216	16.335	0.010	
AC	6.400	1	6.400	2.537	0.172	
BC	11.730	1	11.730	4.649	0.084	
A^2	11.957	1	11.957	4.740	0.081	
B^2	2.808	1	2.808	1.113	0.340	
C^2	22.201	1	22.201	8.8	0.031	
Residual	12.615	5	2.523			
Lack of Fit	9.412	3	3.137	1.958	0.356	not significant
Pure Error	3.203	2	1.601			

Table 5 Model fitting values

No	Model terms	Model fitting values	
		Sophorolipid yield	**Yield Coefficient**
1	Coefficient of the variation	13.480	11.140
2	Multiple regression coefficient (R^2)	0.974	0.983
3	Adjusted R^2	0.926	0.952
4	The signal to noise ratio (adequate precision)	14.981	17.869

Figure 1

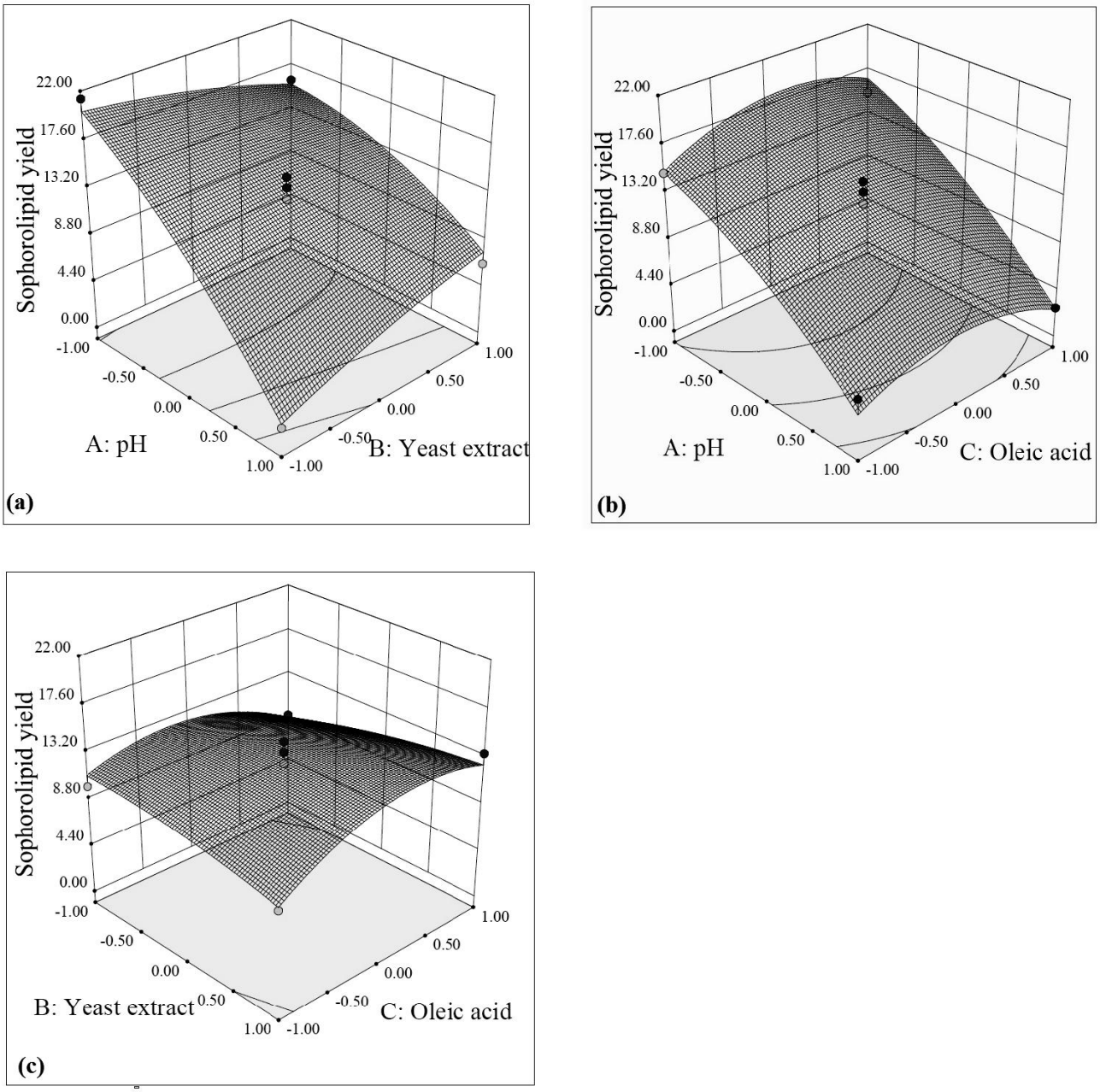

Fig. 1 Three-dimensional response surface plots for sophorolipid yield (g/L)

(a) pH and yeast extract, (b) pH and oleic acid, (c) yeast extract and oleic acid

Figure 2

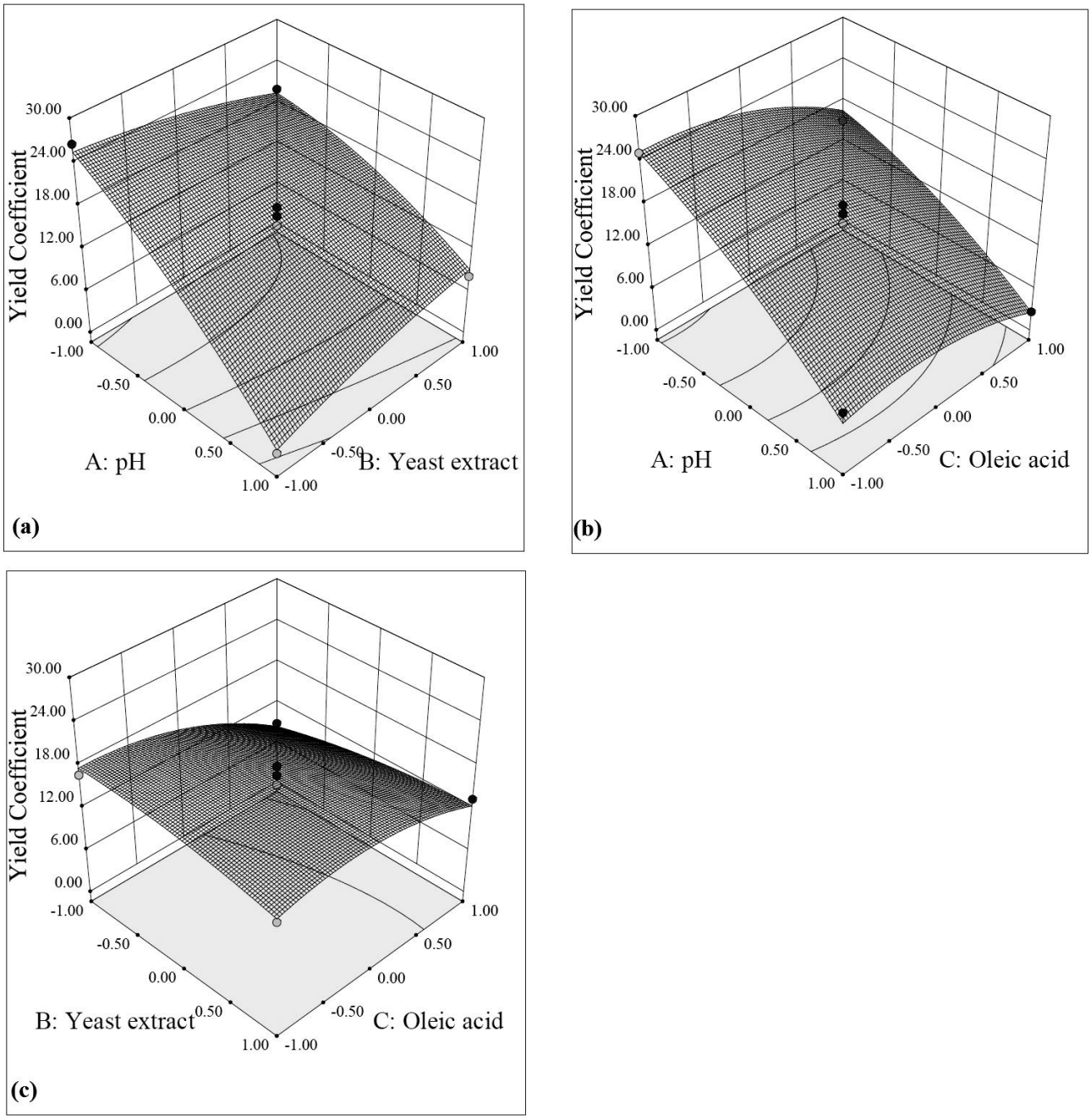

Fig. 2 Three-dimensional response surface plots for yield coefficient

(a)pH and yeast extract, (b) pH and oleic acid, (c) yeast extract and oleic acid

DISCUSSION:

Concentration of sophorolipid obtained in the basal medium was 3.2 g/L, and the corresponding yield coefficient was 1.6. After the placket-burman screening experiments, and optimization by RSM experiments, the maximum concentration of sophorolipid obtained was 18.2 g/L, with a yield coefficient of 30.33. Thus 5.68 fold increases in the sophorolipid yield, and 18.95 fold increases in the yield coefficient (grams of sophorolipids per 100 grams of carbon substrate) was observed. However, the sophorolipid yields obtained during this study were much lower than the highest reported yields in the literature [13,14]. Rau et al [14] have reported high yields > 300 g/L using a total of 440 g/L of carbon source (rapeseed oil 140 g/ L and glucose 300 g/L), which corresponds to a yield coefficient of 68. Similarly Daniel et al [13] has reported high sophorolipid yield of 422 g/L using a total of 600 g/L of substrate (Deprotinised whey concentrate having 200 g/L of deprotinised whey and 400 g/L of rapeseed oil), which corresponds to a yield coefficient of 70.33. However it should be noted that, both Daniel et al[13] and Rau et al [14] had not only used very high substrate concentration, but had also used larger scale bio reactors, where the feeding pattern of substrate, pH, aeration and various other parameters were controlled and maintained at the desired levels. Further, it should be noted that the cultivation process reported by the Daniel et al [13] involves a total of two steps. In the first step, deproteinised whey concentrate (DWC) was used for cultivation of the yeast *Cryptococcus curvatus,* which lead to the breakdown of lactose and production of single cell oil. Second step involved disruption of the cells by passing the cell suspension directly through a high pressure laboratory homogeniser. And after autoclaving, the resulting crude cell extract containing the single-cell oil was served as a substrate for growth of *Candida bombicola ATCC 22214,* where the production of sophorolipids occurred with consumption of single-cell oil and repeated feeding of 400 g rapeseed oil.

There are no reports on the maximum sophorolipid yield or yield coefficient that can be obtained using simple substrates like glucose and oleic acid at a scale of shake flask. The highest yield of sophorolipis, with the use of glucose and oleic acid as carbon sources is reported by Solaiman DKY et al [15] is of 79g/L, with step wise feeding of a total of 60 g/L of oleic acid, and 175 g/L of glucose, thus corresponding to a yield coefficient of 33.61 in a 12L capacity bench top fermentor equipped with pH, and aeration control. Thus, compared to the above results, even though the maximum sophorolipid yield obtained in our study (18.2 g/L) is four times lower than the maximum reported yield (79 g/L), but the yield coefficient obtained in this study (30.33) is comparable (even though our study was carried out at a shake flask scale, where it was not possible to maintain pH, substrate concentration and aeration at desired level) to the maximum reported yield coefficient (33.61), with the use of glucose and oleic acid as substrate.

CONCLUSION:

There is a growing acceptance for the use of statistical experimental designs in biotechnology. The application of statistical design for screening and optimization of process parameters allows quick identification of important factors and interactions between them. In the present study, Box-Behnken design was useful in studying the physicochemical factors that influenced production of sophorolipids by S. bombicola under submerged fermentation at shake flask scale. Similarly, statistical experimental designs can also be applied to optimize the

fermentation parameters at a larger scale. i.e. at lab scale, pilot scale or industrial scale fermentors, where the feeding pattern and the parameters such as pH, aeration and substrate concentrations etc can be controlled and maintained at their optimum levels to obtain not only higher Sophorolipid yields but also to maximize yield coefficients. Scaling up of these optimized operating parameters with the use of a lab-scale fermentor is under progress.

ACKNOWLEDGEMENTS:

We would like to acknowledge ARS culture collection, USA for providing the yeast Starmerella bombicola NRRL Y-17069 as a free gift sample and University Grant Commission (UGC), India for the financial support.

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