/ 3 $ 3 '(25, M E E Y L

On the role of medium components in bio-surfactant production from *Achromobacter xylos GSR21*

Golamari Siva Reddy[1], Kamma Srinivasulu[1], Botlagunta Mahendran[2] and Ronda Srinivasa Reddy[3*]

Centre for Bioprocess Technology, Department of biotechnology, Koneru Lakshmaiah Education Foundation (Deemed to be University), Green Fields, Vaddeswaram, Guntur, Andhrapradesh, India - 522502.

[*]*Corresponding author: siva_bt@kluniversity.in*

ABSTRACT

This paper, for the first time, reports the optimization of the critical medium components for bio-surfactant production from *achromobacter xylos* strain GSR21 using statistical experimental design. Response surface methodology (RSM) was employed to determine the optimal levels of process variables (agar powder, yeast extract, $FeSO_4 7H_2O$, and KH_2PO_4). Central composite design (CCD) of RSM was used to study the four variables at five levels, and bio-surfactant concentration was measured as response. Regression coefficients were calculated by regression analysis, and the model equation was determined. R^2 value for bio-surfactant (g/L) was tested to be 0.7222, indicating that the model fitted well with the experimental results. Verification of the mathematical model was conducted by performing the experiment with the predicted optimized values, and bio-surfactant yield was found to be 9.69 g/L. Validation of the predicted model was fitted 96.9% with the experimental results conducted under the optimum conditions. Agar powder and yeast extract was identified as efficient components for bio-surfactant (*achromobacter xylos* GSR21) production.

Keywords: Achromobacter xylos; Bio-surfactant; Central composite design; Response surface methodology.

1. INTRODUCTION

Surfactants are generally natural intensifiers that are amphiphilic in nature containing both hydrophobic and hydrophilic gatherings, and they are utilized to bring down the interfacial tension between two fluids. [6, 16] Surfactants may function as detergents, wetting agents, emulsifiers, foaming agents, and dispersants.[23] Bio-surfactants derived from microorganisms can be used to replace synthetic surfactants. They are complex particles that can be grouped in terms of structures that involve lipopeptides, glycolipids, polysaccharide-protein buildings, unsaturated fats, and phospholipids.[18]The real points of interest for utilizing bio-surfactants are biodegradability, low toxicity, and possibility of generation from inexhaustible, not-so-costly substrates.[15] Bio-surfactants are primarily utilized for bioremediation to treat hydrocarbon-contaminated destinations and furthermore for oil recuperation. They are likewise utilized as additives for the production of pesticides, medicinal services and beautifiers, mash and paper and nourishment ventures.[8, 11] Microorganisms, including Bacillus subtilis, Bacillus licheniformis, Bacillus pumilis, and Pseudomonas putida, are equipped for delivering bio-surfactants.[1,9,19,21] Lipopeptides derived from Bacillus subtilis are particularly intriguing due to their high surface action and restorative potential.[3,20]

Optimization of medium and fermentation conditions is the first step in bioprocess improvement, and it involves several variables.[5] One factor at a time optimization is an accepted method, however it has numerous weaknesses like more trial runs and time[10]. Response surface method (RSM) is an accumulation of measurable devices to outline and examinations the analyses concentrated on improvement.[17] RSM is effectively utilized to decide the ideal states of the chose factors associated with the procedure.[1,12,24] The fundamental preferred standpoint of utilizing RSM is to assess the cooperation impact of the factors under investigation with the assistance of reaction surface plots produced by the product.

The objective of the current study was to determine the optimal levels of the medium components for bio-surfactant production from *Achromobacter xylos* strain GSR21 by response surface methodology.

2. MATERIALS AND METHODS

2.1 Microorganism

The microorganism (*Achromobacter xylos* GSR21) used in this study was obtained from Environmental Microbiology Laboratory culture collection of the Department of Biotechnology at K L University Andhrapradesh, India. The culture was maintained in LB agar plates incubated at 30°C and sub-cultured at regular intervals. Inoculums were prepared by transferring a loopful of culture to a 100 mL of sterilized Luria Bertani (LB) broth and kept in a rotary shaker incubator at 200 rpm and 30°C for 48 h. All chemicals were of analytical grade and procured from Quality-Control, Hyderabad, India.

2.2 Fermentation

Two percent (W/V) of the seed culture was inoculated in the production media containing (g/L): "glycerol, 5 g; asparagine 1 g; KH_2PO_4, 1 g; $MgSO_4 \times 7H_2O$, 5 g; KCl, 1.0 g; agar powder, 15 g; and 1 mL of trace solution containing (in 1 L of distilled water) $MgSO_4 \times 7H_2O$, 0.5 g, $CuSO_4 \times 5H_2O$, 0.16 g, and $FeSO_4 \times 7H_2O$, 0.015 g" . The initial pH of the medium was adjusted to 8.0.[13] All fermentations were carried out at 30°C in a shaker flask held on a rotary platform shaker at 200 rpm. For statistical optimization experiments, 100 mL of medium was prepared in a 250 mL conical flask according to the central composite design given in Table.1.

2.3 Bio-surfactant precipitation

1.5 mL of fermented broth was collected in a 2 mL Eppendorf tube and centrifuged at 10 000 rpm for 10 minutes. After centrifugation, the supernatant was used for the extraction of bio-surfactant. 6 N HCl was added to the Eppendorf containing supernatant and kept overnight for incubation. Then, the sample was centrifuged at 6 000 rpm for 10 min, and the precipitated bio-surfactant was collected in the form of pellets. The precipitated bio-surfactant was dried in a hot air oven at 80°C overnight, and the weight of the crude bio-surfactant was determined.

2.4 Experimental design

Four medium variables (Agar powder, yeast extract, $FeSO_4.7H_2O$, and KH_2PO_4) were selected for RSM optimization based on preliminary screening studies. The range of level of four variables is given in Table 1. Thirty experiments were carried out according to central composite design (CCD) shown in Table 2.The relationship between the variables and the response is generally represented by the second order polynomial equation (Eqn. 1).

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_4 X_4 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{33} X_3^2 + \alpha_{44} X_4^2 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{14} X_1 X_4 + \alpha_{23} X_2 X_3 + \alpha_{24} X_2 X_4 + \alpha_{34} X_3 \quad \ldots\ldots\ldots (1)$$

Table 1: Range of variable levels for RSM experiment

Factors (g/L)	Symbol	2	1	0	-1	-2
Agar powder	A	70	60	50	40	30
Yeast extract	B	7	6	5	4	3
$FeSO_4.7H_2O$	C	0.06	0.055	0.05	0.045	0.04
KH_2PO_4	D	0.25	0.2	0.15	0.1	0.05

3. RESULTS AND DISCUSSION

3.1 Response surface optimization

Statistical optimization for bio-surfactant production was carried out according to central composite design of RSM using Design expert software. The response, bio-surfactant concentration was estimated for thirty experiments and represented in Table.2.The response data were subjected to regression analysis to estimate regression coefficient. The estimated coefficients are shown n Table 3. A second order polynomial equation (Final Equation in Terms of Coded Factors) (Eqn. 2) and Final Equation in Terms of Actual Factors (Eqn.3) for bio-surfactant production was constructed by using the coefficients.

$$Y_{Biosurfactant\,(\frac{g}{L})} = +8.04 + 0.60A + 0.23B - 0.41C - 0.23D - 0.72AB + 0.21AC + 0.21AD - 0.017BC + 0.29BD + 0.72CD + 0.27A^2 - 0.44B^2 - 0.42C^2 + 0.043D^2 \quad \ldots\ldots\ldots (2)$$

Final Equation in Terms of Actual Factors:

$$Y_{Biosurfactant\,(\frac{g}{L})} = 1.96086 - 0.014745 \times Agarpowder + 1.95536 \times Yeast\ extract + 224.80208 \times FeSO_4.7H_2O - 52.13854 \times KH_2PO_4 - 0.018109 \times Agarpowder \times Yeast\ extract + 1.04687 \times Agarpowder \times FeSO_4.7H_2O + 0.10406 \times Agarpowder \times KH_2PO_4 - 0.84375 \times Yeast\ extract \times FeSO_4.7H_2O + 1.47188 \times Yeast\ extract \times KH_2PO_4 + 720.62500 \times FeSO_4.7H_2O \times KH_2PO_4 + 0.000673177 \times (Agarpowder)^2 - 0.11112 \times (Yeast\ extract)^2 - 4219.79167 \times (FeSO_4.7H_2O)^2 + 4.30208 \times (KH_2PO_4)^2 \quad \ldots\ldots\ldots (3)$$

The adequacy of the model was verified using analysis of variance (ANOVA), and the results are shown in Table 3. The Model F-value of 2.79 implied that the model was significant. There is only a 2.92% chance that a "Model F-Value" this large could occur due to noise. High value of F-test for regression indicated that the model was well fitted, which can explain the variation observed in bio-surfactant concentration in terms of the designed levels of variables. Probability value (p<0.0500) is usually used to check the statistical significance of the parameters. Results represented in Table 3 explained that the individual effect of agar powder (A), agar powder*yeast extract (AB), $FeSO_4.7H_2O$* KH_2PO_4 (CD) and square effect of yeast extract (B^2) and $FeSO_4.7H_2O$ (C^2) were significant in bio-surfactant production. R^2 was 0.7222, and this value indicated that the model was fitted for 72.2% of bio-surfactant production. These results showed that the model can satisfactorily explain both linear and square effects of the process variables.

The combined effect of agar powder and yeast extract is shown in Figure 1a. Maximum bio-surfactant production (10.2 g/L) was achieved at a low level of yeast extract (4.53 g/L). There was a significant increase in the

product concentration when agar powder concentration increased from 30 g/L to 70 g/L.[14,22] It was reported that agar powder was the most suitable carbon source for bio-surfactant production from *glycolipid* in comparison with the other carbohydrates studied. Several researchers concluded that the presence of yeast extract with a low concentration can increase bio-surfactant productivity.[7,22] Supplementation of yeast extract (4 g/L) in the production medium can enhancebio-surfactant production as the amino acids are required for the formation of the glycolipid bio-surfactant from *Achromobacter xylos* GSR21.[4,22] It was also reported that a low level of yeast extract resulted in the enhancement of bio-surfactant production.

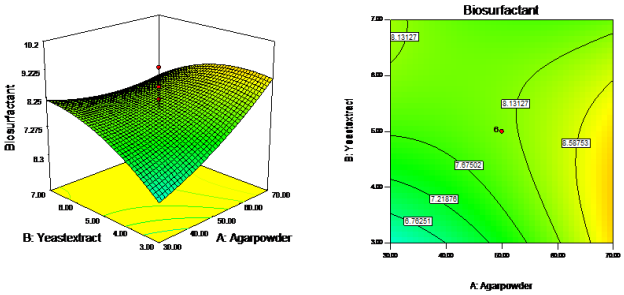

Figure 1a: 3D and contour surface plots showing the impact of agar powder (A) and yeast extract (B) on bio-surfactant production.

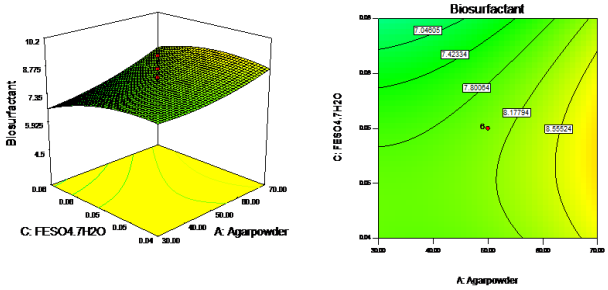

Figure 1b. 3D and contour surface plots showing the impact of agar powder (A) and $FeSO_4.7H_2O$ (C) on bio-surfactant production.

As shown in Figure 1b, increased use of agar powder and $FeSO_4.7H_2O$ led to improvement of bio-surfactant production. It was observed that the $FeSO_4.7H_2O$ in the medium had a significant role in productivity. When agar powder concentration increased from low to high level, the productivity was also increased whereas increase in concentration of KH_2PO_4 did not have noticeable impact on bio-surfactant production (Figure 1c).

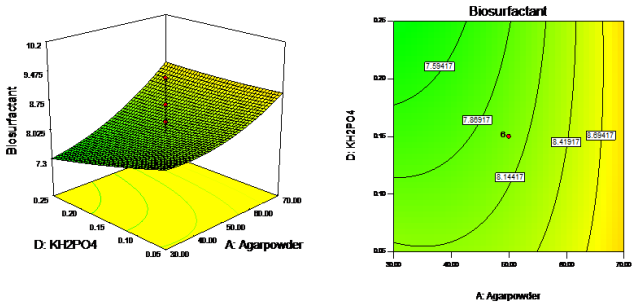

Figure 1c. 3D and contour surface plots showing the impact of agar powder (A) and KH_2PO_4 (D) on bio-surfactant production.

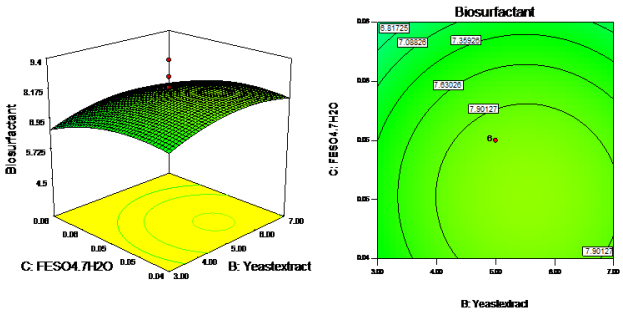

Figure 1d. 3D and contour surface plots showing the impact of yeast extract (B) and $FeSO_4.7H_2O$ (C) on bio-surfactant production.

As shown in Figure 1d, bio-surfactant production decreased when the yeast extract increased from low to high level, implying that 4.53 g/L is sufficient for optimum productivity. Howegver, the productivity increased when the concentration of $FeSO_4.7H_2O$ increased from low to high level.

As shown in Figure 1e, bio-surfactant production decreased when yeast extract concentration increased from low to high levels, whereas KH_2PO_4 had a negligible impact. The productivity of bio-surfactant increased when the concentration of ferrous sulphate increased from low to high levels (Figure 1f).

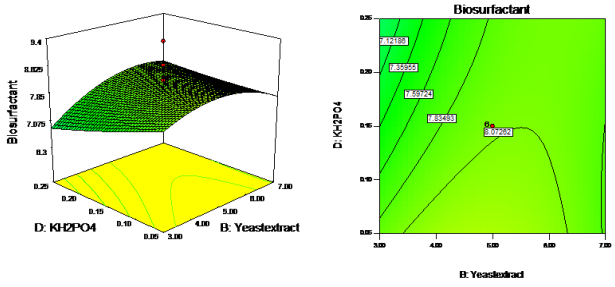

Figure 1e.3D and contour surface plots showing the impact of yeast extract (B) and KH_2PO_4 (D) on bio-surfactant production.

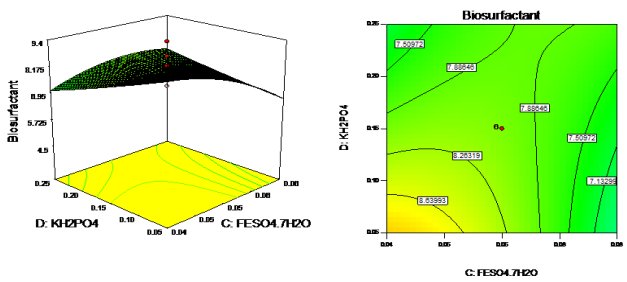

Figure 1f. 3D and contour surface plots showing the impact of $FeSO_4.7H_2O$ (C) and KH_2PO_4 (D) on bio-surfactant production.

Point prediction tool of Design Expert software was used to determine the optimal level of each variable in the process. The maximum bio-surfactant concentration (10.20 g/L) was predicted by the software at optimal levels of ingredients: agar powder - 90 g/L, yeast extract - 5 g/L, $FeSO_4.7H_2O$ - 0.055 g/L and KH_2PO_4-0.15 g/L.

Table 2. Central composite design matrix with experimental and predicted values of bio-surfactant produced from *Achromobacter xylos* strain GSR21

Run order	Medium Components				Bio-surfactant (g/L)		
	A	B	C	D	Experimental	Predicted	Residual
1	30	3	0.04	0.05	7.33	7.98	-0.65
2	70	3	0.04	0.05	8.53	9.79	-1.26
3	30	7	0.04	0.05	8.67	9.34	-0.67
4	70	7	0.04	0.05	9.33	8.26	1.07
5	30	3	0.06	0.05	5.33	5.33	0.00
6	70	3	0.06	0.05	9.33	7.98	1.35
7	30	7	0.06	0.05	7.33	6.63	0.70
8	70	7	0.06	0.05	5.33	6.38	-1.05
9	30	3	0.04	0.25	5.33	5.08	0.25
10	70	3	0.04	0.25	7.33	7.73	-0.40
11	30	7	0.04	0.25	6.58	7.62	-1.04
12	70	7	0.04	0.25	6.57	7.37	-0.80
13	30	3	0.06	0.25	4.55	5.32	-0.77
14	70	3	0.06	0.25	8.67	8.80	-0.13
15	30	7	0.06	0.25	8.25	7.79	0.46
16	70	7	0.06	0.25	9.33	8.38	0.95
17	10	5	0.05	0.15	8.53	7.92	0.61
18	90	5	0.05	0.15	10.2	10.32	-0.12
19	50	1	0.05	0.15	6.35	5.79	0.56
20	50	9	0.05	0.15	6.67	6.73	-0.06
21	50	5	0.03	0.15	8.67	7.17	1.50
22	50	5	0.07	0.15	4.53	5.53	-1.00
23	50	5	0.05	-0.05	8.67	8.66	0.01
24	50	5	0.05	0.35	8.25	7.76	0.49
25	50	5	0.05	0.15	7.33	8.04	-0.71
26	50	5	0.05	0.15	9.33	8.04	1.29
27	50	5	0.05	0.15	7.33	8.04	-0.71
28	50	5	0.05	0.15	8.25	8.04	0.21
29	50	5	0.05	0.15	7.33	8.04	-0.71
30	50	5	0.05	0.15	8.67	8.04	0.63

Table 3. ANOVA statistics for bio-surfactant production from *Achromobacter xylos* GSR21

Factors	Sum of Squares	df	Mean Squares	F Value	p-value	Significance
Model	48.10	14	3.44	2.79	0.0292	significant
A-Agarpowder	8.63	1	8.63	6.99	0.0184	significant
B-Yeastextract	1.32	1	1.32	1.07	0.3172	
C- $FeSO_4.7H_2O$	4.03	1	4.03	3.26	0.0909	significant
D- KH_2PO_4	1.22	1	1.22	0.99	0.3359	
AB	8.40	1	8.40	6.81	0.0198	significant
AC	0.70	1	0.70	0.57	0.4625	
AD	0.69	1	0.69	0.56	0.4651	
BC	0.00	1	0.00	0.00	0.9523	
BD	1.39	1	1.39	1.12	0.3058	
CD	8.31	1	8.31	6.74	0.0203	significant
A^2	1.99	1	1.99	1.61	0.2235	
B^2	5.42	1	5.42	4.39	0.0535	significant
C^2	4.88	1	4.88	3.96	0.0652	significant
D^2	0.05	1	0.05	0.04	0.8420	
Residual	18.50	15	1.23			
Lack of Fit	14.89	10	1.49	2.06	0.2203	not significant
Pure Error	3.62	5	0.72			
Cor Total	66.61	29				

3.2 Model validation

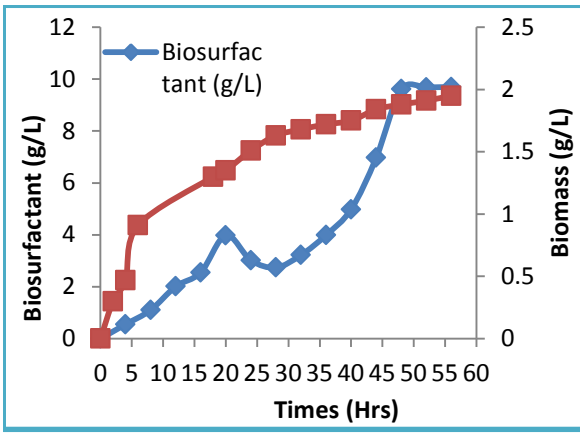

Figure 2. Time course profile of bio-surfactant and biomass production from Achromobacter xylos GSR 21. Note that the data were generated under predicted process conditions

In the validation experiment, maximum bio-surfactant concentration of 9.69 g/L was obtained. The time course profile of bio-surfactant and biomass production from *Achromobacter xylos* GSR21 on the basis of predicted optimal levels of the medium components is shown in

To check the accuracy of the predicted model, experiments were carried out under the predicted optimal process conditions: agar powder - 90 g/L, yeast extract - 5 g/L, $FeSO_4.7H_2O$ - 0.055 g/L, and KH_2PO_4-0.15 g/L.

concentration of 9.69 g/L was obtained. The time course profile of bio-surfactant and biomass production from *Achromobacter xylos* GSR21 on the basis of predicted optimal levels of the medium components is shown in Figure 2. The validation result indicated that the predicted model was fitted 96.9% with the experimental results.

Figure 2. The validation result indicated that the predicted model was fitted 96.9% with the experimental results.

To check the accuracy of the predicted model, experiments were carried out under the predicted optimal process conditions: agar powder - 90 g/L, yeast extract - 5 g/L, $FeSO_4.7H_2O$ - 0.055 g/L, and KH_2PO_4-0.15 g/L.

4. CONCLUSION

5.

Response surface methodology was applied to optimize the four media components to enhance the bio-surfactant production. Four variables (agar powder, yeast extract, $FeSO_4.7H_2O$, and KH_2PO_4) were examined according to central composite design of RSM. Surface

plots were made, and the optimized values obtained for the maximum production of bio-surfactant were: agar powder - 90 g/L, yeast extract - 5 g/L, $FeSO_4.7H_2O$ - 0.055 g/L, and KH_2PO_4-0.15 g/L. Validation of the experiment was performed and it indicated that the model was well fitted with the experimental results. The application of RSM would simplify the process optimization for bio-surfactant production, certainly serving as a paradigm for streamlined exploration into variables of interest.

AUTHORS' CONTRIBUTIONS

GSR and BM conceived the idea pertaining to the study. GSR carried out the laboratory analysis. GSR, BM, and RSR participated in the experimental design and coordination and drafting of the manuscript. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors are grateful to Sri. K Satyanarayana Garu, president, K L University for supporting this research work. The authors are also thankful to Prof. L S S Reddy, Vice Chancellor for giving permission and Dr. K. Srinivasulu, HOD-BT for his encouragement during this work.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Abbasi H., Sharafi H., Alidost L., Bodagh A., Zahiri H. S, Noghabi K. A. Response surface optimization of bio-surfactant produced by *Pseudomonas aeruginosa* MA01 isolated from spoiled apples. Prep. Biochem. Biotechnol., 43: 398-414, 2013.
2. Bas D., Boyaci I. H. Modeling and optimization I: Usability of response surface methodology. J. Food Eng., 78: 836-845, 2007.
3. Besson F., Michel G. Biosynthesis of iturin and surfactin by *Bacillus subtilis*. Evidence for amino acid activating enzymes. Biotechnol. Lett., 14: 1013-1018,1992.
4. Casas J. A., García-Ochoa F. Sophorolipid production by *Candida bombicola*: medium composition and culture methods. J. Biosci. Bioeng., 88: 488-494,1999.
5. Chakravarti R., Sahai V. Optimization of compactin production in chemically defined production medium by *Penicillium citrinum* using statistical methods. Process Biochem., 38: 481-486,2002.
6. Desai J. D, Banat I. M. Microbial production of bio-surfactants and their commercial potential. Microbiol. Mol. Biol. Rev., 61: 41-64, 1997.
7. Gandhimathi R., Seghal Kiran G., Hema T. A. Selvin J, Rajeetha Raviji T., Shanmughapriya S. Production and characterization of lipopeptide bio-surfactant by a sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. Bioprocess Biosyst. Eng., 32: 825-835, 2009.
8. Ghojavand H., Vahabzadeh F., Mehranian M., Radmehr M., Shahraki K. A., Zolfagharian F., Emadi M. A., Roayaei E. Isolation of thermotolerant, halotolerant, facultative bio-surfactant producing bacteria. Appl. Microbiol. Biotechnol., 80: 1073-1085, 2008.
9. Guerra de Oliveira J., Garcia-Cruz C. H. Properties of a bio-surfactant produced by *Bacillus pumilus* using vinasse and waste frying oil as alternative carbon sources. Braz. Arch. Biol. Technol., 56: 155-160,2013.
10. Haaland P. D. 1989. Experimental design in biotechnology. Dekker, New York.
11. Hickey A. M., Gordon M., Dodson A. D. W., Kelly C. T., Doyle E. M. Effect of surfactants on fluoranthene degradation by *Pseudomonas alcaligenes* PA-10. Appl. Microbiol. Biotechnol., 74: 851-856,2007.
12. Khayati G., Gilani H. G., Kazemi M. The effect of olive cake types on lipase production by isolated Rhizopus sp. and process statistical optimization. J. Biosci. Biotechnol., 2: 45-55, 2013.
13. Kim H., Yoon B., Lee C., Suh H., Katsuragi T., Tani Y. Production and properties of a lipopeptide bio-surfactant from *Bacillus subtilis* C9. J. Ferm. Bioeng., 84: 41-46,1997.
14. Makkar R. S., Cameotra S. S. Effects of various nutritional supplements on bio-surfactant production by a strain of *Bacillus subtilis* at 45°C. J. Surf. Deter., 5: 11-18, 2002.
15. Mohan P. K, Mukhla G., Yanful E. K. Biokinetics of biodegradation of surfactants under aerobic, anoxic and anaerobic conditions. Water Res., 40: 533-540, 2006.
16. Mulligan C. N. Environmental application for bio-surfactants. Environ. Poll., 133: 183-198,2005.
17. Myers R. H., Montgomery D. C. 1995. Response surface methodology, process and product optimization using design experiments. John Wiley & Sons, New York.
18. Neto D. M., Meira J. A., de Araujo J. M., Mitchel D. A., Krieger N. Optimization of the production of rhamnolipids by *Pseudomonas aeruginosa* UFPEDA 614 in solid-state culture. Appl. Microbiol. Biotechnol., 81: 441-448,2008.
19. Priya R., Suresh Kumar S., Gupta R. Concomitant production and downstream processing of alkaline protease and bio-surfactant from *Bacillus licheniformis* RG1:Bioformulation as detergent additive. Process Biochem., 40: 3352-3359, 2005.
20. Sandrin C., Peypoux F., Michel G. Coproduction of surfactin and iturin A lipopeptides with surfactant and antifungal properties by *Bacillus subtilis.* Biotechnol. Appl. Biochem., 12: 370–375, 1990.
21. Sanket J., Chirag B., Anjana J. D. Production of bio-surfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. Bioresour. Technol., 99: 4603-4608, 2008.
22. Sasidharan Satheesh Kumar , Govindasamy Sharmila, Chandrasekaran Muthukumaran, Krishnamurthi Tamilarasan and Margavelu Gopinath., Statistical optimization of critical medium components for bio-surfactant production by *Bacillus subtilis*., *J. BioSci. Biotechnol.* 4(2): 123-129, 2015.
23. Singh A., Van Hamme J. D., Ward O. P. Surfactants in microbiology and biotechnology: Part 2. Application aspects. Biotechnol. Adv., 25: 99–121, 2007.
24. Tamilarasan K., Dharmendira Kumar M. Optimization of medium components and operating conditions for the production of solvent-tolerant lipase by *Bacillus sphaericus* MTCC 7542. Afr. J. Biotechnol., 10: 15051-15057, 2011.