International Journal of Advanced Biotechnology and Research ISSN 0976-2612,
Vol 2, Issue 4, 2011, pp 422-430 **http://www.bipublication.com**

APPLICATION OF STATISTICAL DESIGN TO THE OPTIMIZATION OF CULTURE MEDIUM FOR BIOMASS PRODUCTION BY *EXIGUOBACTERIUM* SP. HM 119395

Panneerselvam Kiruthika[1], S. Durairaju Nisshanthini[2], Ayyaswamy Saraswathi[2], Jayaraman Angayarkanni[2], Rasappan Rajendiran[3]

[1]Research and Development Centre, Bharathiar University, Coimbatore 641 046, India.
[2]Cancer Therapeutics Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore-641 046, India.
[3]Poultech Agroresearch Centre, Namakkal, India.

ABSTRACT
The central composite design was developed to search for an optimal medium for the growth of *Exiguobacterium* sp. The effect of various media components, such as sucrose, peptone, yeast extract, sea water, glycerol, pH, incubation periods and inoculum size were examined. The first-order model based on Plackett-Burman design showed that sea water, pH and sucrose and glycerol influenced the growth of the examined bacteria. The second-order polynomial regression confirmed that maximum biomass production was achieved by the combination of pH (8.7), sea water (350 ml), sucrose (2.0 g) and glycerol (0.5 ml) with the predicted maximum biomass production of 0.8116 O.D of fermented media. The organism produced 0.807 O.D. confirming the validity.

Key words: *Exiguobacterium sp., Media optimization, Growth, Plackett-Burman design and Central composite design*

INTRODUCTION

During the past two decades, research on marine bacteria has highlighted the tremendous potential of these microorganisms as source of new bioactive secondary metabolites [2, 17]. Although marine organisms do not have a significant history of use in traditional medicine, in the last years marine microorganisms have become an important point of study in search of novel microbial products showing antimicrobial activities, antiviral, immunosuppressives, enzyme inhibitor metabolites, receptor antagonists, antitumor activities and anticoagulant properties [3,8,13]. The most of the antibiotic producing marine bacteria were pigmented [11]. Media composition and growth conditions influence the culture growth thus the antibacterial performance. Antibacterial activity of culture can be increased with proper optimized media composition.

Traditional methods for optimization are "one-factor-at-a-time" techniques. Unfortunately, this approach frequently fails to identify the variables that give rise to the optimum response because the effects of factor interactions are not taken into account in such procedures [5]. Moreover, they require a considerable amount of work and time [18]. So, effective problem solving methods are preferred. Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and

give an estimate of combined effect of these variables on final results. Response surface methodology (RSM) is one such technique based on the fundamental principles of statistics, such as randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner [7].

In this study, statistical optimization of medium constituents was employed to enhance growth of *Exiguobacterium* sp. To the best of our knowledge, there is not enough information concerning optimum nutritional requirements for *Exiguobacterium* sp. using statistical experimental designs. In the first step, a Plackett–Burman design was used to determine the likely effects of nine possible medium variables on growth of *Exiguobacterium* sp. Subsequently, the concentrations of the medium components that had significant effects were optimized using a central composite design and response-surface analyses.

[II] MATERIALS AND METHODS

2.1 Chemicals

Pure and analytical grade chemicals were used in all experiments, including media preparation for growth. Peptone, yeast extract, and other chemicals were purchased from Hi-Media Co, Mumbai, India.

2.2 Organism

Exiguobacterium sp. was isolated from coastal surface sea water from Marina, Tamil Nadu, India on January 2010. The bacterium was identified using the 16S rDNA gene sequence analysis and was submitted to Genbank under the accession number HM 119395. It was propagated on Sea water yeast extract peptone medium (SYEP) agar slants at 35°C for 3 days and subcultured monthly.

2.3 Optimization of process parameters

2.3.1 Identification of suitable variables using Plackett – Burman (PB) design

The Plackett – Burman experimental design identifies the critical nutrient and process variables required for elevated growth by screening n variables in $n + 1$ experiments [12]. The variables chosen for the present study were sucrose (g/L), peptone (g/L), yeast extract (g/L), sea water (mL), glycerol (mL/L), pH, incubation periods (days) and inoculum size (mL/L) in the culture medium. Eight assigned variables and three unassigned variables (commonly referred as dummy variables) were screened in PB design of 12 experiments. Dummy variable are used to estimate experimental errors in data analysis [15]. Eight factors consisting of medium components and operating conditions prepared at two levels –1 for low level and +1 for high level (Table-1).

Table1: Actual values of the process variables for 1000ml of medium

Process variables	Sucrose (g)	Peptone (g)	Yeast Extract (g)	Sea water (mL)	Glycerol (mL)	pH	Incubation periods (Days)	Inoculum size (mL)
Low level (-1)	5.0	0.5	0.5	100	0.5	4.0	1	0.5
High Level (+1)	20.0	5.0	5.0	1000	3.0	8.0	3	2.0

The experimental design for the screening of the variables was presented in Table 2. PB experimental design is based on the first order model as given in equation 1.

$Y= \beta_0 + \Sigma \beta i\, xi$ …… (1)

Where, Y is the response (pigment production), β_0 is the model intercept, β_i is variable estimates and x_i are independent variables. All experiments were carried out in duplicate and the averages of growth of the organism were taken as responses. The variables whose confidence levels were higher than 90% were considered that significantly influences the growth and pigment production of the organism

Table 2: Plackett-Burman design of 12 runs for coded values of 11 variables of biomass production

Run	Sucrose (g)	Peptone (g)	Yeast Extract (g)	Sea water (mL)	Glycerol (mL)	pH	Incubation periods (days)	Inoculum size (mL)	DV-1	DV-2	DV-3	Experimental OD value	Predicted OD value
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	0.057	0.022
2	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	0.092	0.093
3	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	0.030	0.018
4	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	1.395	1.418
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	0.028	0.027
6	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	1.287	1.300
7	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	1.510	1.487
8	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	0.094	0.129
9	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	1.460	1.425
10	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	1.252	1.253
11	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	1.365	1.388
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.025	0.037

Sign '+1' is for high concentration of variables and '-1'is for low concentration of variables

2.3.2 Central composite design for optimization of media for biomass production

Once critical factor were identified via screening, the central composite design (CCD) was proceeded to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with pigment production as response. The effect of the parameters pH, sea water, sucrose and glycerol on the cell biomass was studied at five experimental levels: -α, -1, 0, +1, +α, where $\alpha = 2^{n/4}$, here n was the number of variables and 0 corresponded to the central point. The levels of factors used for experimental design are given in Table 3 and design of factorial, axial and center points were noted in Table 4.

The linear quadratic model with 4 variables expressed as:

$Y=\beta o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_{11} + \beta_{22}X_{22} + \beta_{33}X_{33} + \beta_{44}X_{44} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4$------ (2)

Where y is the measured response, β_0 is the intercept term, β_1, β_2, β_3, β_4 are linear coefficient, β_{11}, β_{22}, β_{33}, β_{44} are quadratic coefficient, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are interaction coefficient and X_1, X_2 , X_3, X_4 are coded independent variables.

Table 3: The coded and actual values of the variables in central composite design

Name	Unit	-α	-1	0	+1	+α
pH		4.5	6	7.5	9	10.5
Sea water	mL/L	350	500	650	800	950
Sucrose	g/L	2.0	4.0	6.0	8.0	10.0
Glycerol	mL/L	0.05	0.2	0.35	0.5	0.65

Table 4: Central composite design (CCD) of factors in coded value for optimization of process variables

Trials	Type	pH	Sea water (mL)	Sucrose (g)	Glycerol (mL)	Observed biomass production (OD)	Predicted biomass production (OD)
1	Factorial	-1	-1	-1	-1	0.520	0.544
2	Factorial	1	-1	-1	-1	0.630	0.626
3	Factorial	-1	1	-1	-1	0.464	0.489
4	Factorial	1	1	-1	-1	0.574	0.543
5	Factorial	-1	-1	1	-1	0.473	0.470
6	Factorial	1	-1	1	-1	0.540	0.531
7	Factorial	-1	1	1	-1	0.582	0.552
8	Factorial	1	1	1	-1	0.567	0.584
9	Factorial	-1	-1	-1	1	0.544	0.535
10	Factorial	1	-1	-1	1	0.569	0.598
11	Factorial	-1	1	-1	1	0.459	0.467
12	Factorial	1	1	-1	1	0.489	0.501
13	Factorial	-1	-1	1	1	0.419	0.448
14	Factorial	1	-1	1	1	0.505	0.489
15	Factorial	-1	1	1	1	0.503	0.516
16	Factorial	1	1	1	1	0.553	0.528
17	Axial	-2	0	0	0	0.377	0.352
18	Axial	2	0	0	0	0.430	0.447
19	Axial	0	-2	0	0	0.607	0.590
20	Axial	0	2	0	0	0.565	0.575
21	Axial	0	0	-2	0	0.567	0.544
22	Axial	0	0	2	0	0.483	0.498
23	Axial	0	0	0	-2	0.625	0.634
24	Axial	0	0	0	2	0.586	0.570
25	Center	0	0	0	0	0.552	0.544
26	Center	0	0	0	0	0.588	0.544
27	Center	0	0	0	0	0.510	0.544
28	Center	0	0	0	0	0.517	0.544
29	Center	0	0	0	0	0.564	0.544
30	Center	0	0	0	0	0.530	0.544
31	Center	0	0	0	0	0.550	0.544

2.4 Estimation of cell growth

Cell growth was monitored by absorbance measurements of bacterial cultures at 600 nm optical density (OD 600), which were obtained using a Shimadzu UV160U Spectrophotometer and referenced to uninoculated sterile medium [10].

[III] RESULTS AND DISCUSSION

3.1 Screening the significant variables on biomass production

Variable/ Term	Main Effect	Coefficients	SE Coefficients	t-value	p-value	Confidence level %
Constant		0.71625	0.01253	57.18	0.000	100
Sucrose	-0.06217	-0.03108	0.01253	-2.48	0.089*	91.1*
Peptone	0.00483	0.00242	0.01253	0.19	0.859	14.1
Yeast Extract	0.02517	0.01258	0.01253	1.00	0.389	61.1
Sea water	0.09383	0.04692	0.01253	3.75	0.033*	96.7*
Glycerol	-0.04883	-0.02442	0.01253	-1.95	0.146*	85.4*
pH	1.32383	0.66192	0.01253	52.84	0.000*	100*
Incubation periods	0.00283	0.00142	0.01253	0.11	0.917	8.3
Inoculum size	0.01917	0.00958	0.01253	0.76	0.500	50

Table 5: Statistical analysis of Plackett-Burman design on biomass production by *Exiguobacterium* sp.

R^2 = 99.89% R^2(adj) = 99.61%; SE- Standard error, t – student's test, p – corresponding level of significance, *- significant

The PB results (Table 2) indicated that there was a variation of growth and pigment production in the twelve trials in the range from 0.025 to 1.51 OD at 600nm. The data on biomass yield was subjected to statistical analysis of Plackett-Burman design using MINITAB 15.0 software (Minitab Ltd., Coventry CV32TE, UK) to estimate t-value, p-value and confidence level. The student's t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. On analysis of regression coefficients and t-value of 8 factors were presented in Table 5.

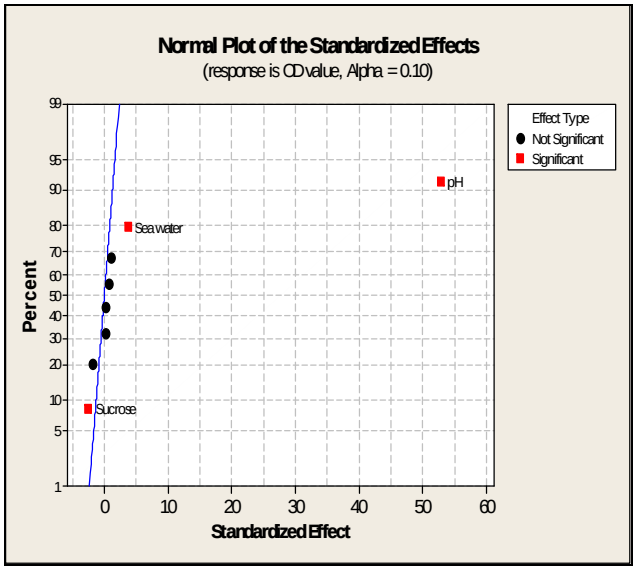


Fig. 1: Normal plot chart of the standardized effects of process variables for biomass production

Among the 8 factors, pH, sea water, sucrose and glycerol showed a positive sign of the effect on biomass production, all other factors shown a negative sign of the effect. When the sign of the effect of the tested variable is positive, the biomass production is greater at a high level of the parameter, and when negative, the biomass production is greater at a low level of the parameter [6]. The coefficient of determination, R^2, was found to be 0.9989 which implied that the sample variation of 99.89% for biomass production was attributed to the independent variables. Meanwhile, the coefficient of determination (adjusted R^2) was calculated to be 0.9961, indicating that a good agreement between the experimental and predicted values of biomass production. On the basis of the confidence level, a normal plot chart of the standardized effects of process variables showing the dominance of the individual variables is shown in Fig. 1 and Table 5 which represented as sea water (confidence level – 96.7%), pH (confidence level - 100%) and sucrose (confidence level – 91.2%) were found as significant factors and glycerol (confidence level – 85.5%) as most important factors influences the biomass production. When marine bacteria were grown in liquid yeast extract peptone glucose and sucrose yeast extract peptone medium they exposed good growth after 48 h in presence of natural seawater or artificial seawater [4, 14]. These key variables were selected for further optimization using RSM.

3.2 Central composite design for optimization of media for biomass production

In CCD, variations of biomass production in the thirty one trials were ranged from 0.377 to 0.630 OD (Table 4). The coefficients t and p values for linear, quadratic and combined effects are given in the Table 6 at 90% significance level. The p-values were used as a tool to confirm the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between the variable. The smaller p-value indicates more significance in the corresponding

coefficient [16]. It was observed that the coefficient for overall effect of the variables had high significance (p = 0.000) on biomass production. The individual effect of pH (p = 0.001), glycerol (p=0.015) and sucrose (p=0.070) and the interaction effect of pH versus pH (0.000), seawater versus sucrose (p=0.00), glycerol versus glycerol (0.017) and seawater versus seawater (0.099) are found the most significant factor on biomass production. The interaction effect of sucrose versus sucrose, pH versus sea water, pH versus sucrose, pH versus glycerol, sea water versus glycerol, sucrose versus glycerol did not have significant influence on biomass production.

The correlation coefficient (R^2 = 87.73%) and adjusted coefficient (R^2 (adjusted) = 77.00%) were also high, which indicates a high significance of the experiments [19]. The equation resulted in an empirical model that relates the measured response to the independent variables of the experiment.

$$Y = 0.5444 + 0.023708*X_1 - 0.00387*X_2 - 0.01145*X_3 - 0.01612*X_4 - 0.03615*X_1^2 + 0.009466*X_2^2 - 0.005784*X_3^2 + 0.01434*X_4^2 - 0.00706X_1X_2 - 0.00543X_1X_3 - 0.00506\ X_1X_4 + 0.03406\ *X_2*X_3 - 0.003562*X_2X_4 - 0.00343*X_3X_4 \text{------- (3)}$$

Where Y is the predicted response of intracellular pigment production, X_1, X_2, X_3 and X_4 were the coded values of pH, sea water, sucrose and glycerol respectively.

The ANOVA of the regression model demonstrates that the model is highly significant, this is evident from the calculated F-value (F-model =8.17) and probability value (p = 0.000). It is evident that the linear (p = 0.002) quadratic effect (p = 0.000) and interaction effect (p=0.011) of the variables had greater influence on intracellular pigment production (Table 7). The F value of model implies that model was significant [1, 9].

Table 6: Estimated Regression Coefficients of central composite design for biomass production

Term		Coefficients	SE Coefficients	t-value	p-value
Constant		0.544429	0.010913	49.888	0.000*
pH	X_1	0.023708	0.005894	4.023	0.001*
Sea water	X_2	-0.003875	0.005894	-0.657	0.520
Sucrose	X_3	-0.011458	0.005894	-1.944	0.070*
Glycerol	X_4	-0.016125	0.005894	-2.736	0.015*
pH*pH	X_1*X_1	-0.036159	0.005399	-6.697	0.000*
Sea water*Sea water	X_2*X_2	0.009466	0.005399	1.753	0.099
Sucrose*Sucrose	X_3*X_3	-0.005784	0.005399	-1.071	0.300
Glycerol*Glycerol	X_4*X_4	0.014341	0.005399	2.656	0.017*
pH*Sea water	X_1*X_2	-0.007062	0.007218	-0.978	0.342
pH*Sucrose	X_1*X_3	-0.005437	0.007218	-0.753	0.462
pH*Glycerol	X_1*X_4	-0.005062	0.007218	-0.701	0.493
Sea water*Sucrose	X_2*X_3	0.034062	0.007218	4.719	0.000*
Sea water*Glycerol	X_2*X_4	-0.003562	0.007218	-0.494	0.628
Sucrose*Glycerol	X_3*X_4	-0.003438	0.007218	-0.476	0.640

R^2 = 87.73% R^2(adj) = 77.00%; SE- Standard error, t – student's test, p – corresponding level of significance, * Significant

Table 7: Analysis of variance (ANOVA) for regression model on effect of independent variables on biomass production

Source	Degree of Freedom	Sum of square	Adjusted Sum of Square	Mean of Square	F-value	p-value
Regression	14	0.095389	0.095389	0.006813	8.17	0.000
Linear	4	0.023242	0.023242	0.005810	6.97	0.002
Square	4	0.051510	0.051510	0.012877	15.45	0.000

Interaction	6	0.020637	0.020637	0.003440	4.13	0.011
Residual Error	16	0.013338	0.013338	0.000834		
Lack-of-Fit	10	0.008823	0.008823	0.000882	1.17	0.441
Pure Error	6	0.004516	0.004516	0.000753		
Total	30	0.108727				

F – Fishers's function, p – corresponding level of significance

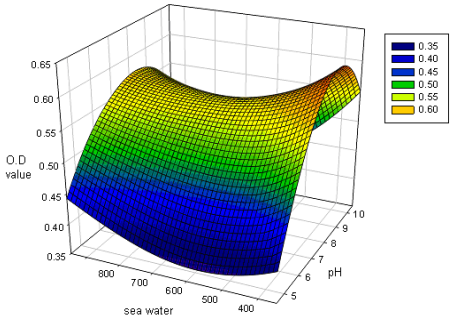
Each figure presents the effect of two factors on biomass production, while the third factor was held at the middle level. These data revealed that the biomass production would increase as pH increase and the minimal amount of sea water, but further increases in these factors after the optimal point would reverse the trend (Fig. 2a). Similar effects on pigment production were observed for pH and sucrose (Fig. 2b). In Fig. 2c, interaction effect of pH and glycerol also results in maximum pigment production. The interaction effect of sea water and sucrose revealed pigment production increased upto 500 mL of seawater and 4.0 g of sucrose (Fig. 2d). Fig. 2e indicates that decrease in sea water and glycerol level will increase in pigment production. In Fig.2f reveals that 2.0 to 4.0 g of sucrose and 0.2 to 0.5 mL of glycerol exhibited the highest pigment production.

3.3 Validation of the Model

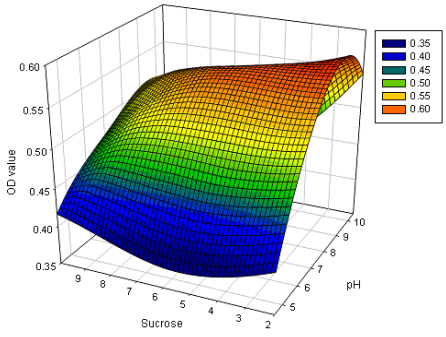
The response optimizer in MINITAB 15.0 software was used to find the optimum value of the variables for maximum biomass production by indigenous isolate of *Exiguobacterium* sp. The optimum value of the variables in actual unit was predicted as pH (8.7), sea water (350mL), sucrose (2.0g) and glycerol (0.5mL) with the predicted maximum biomass production of 0.8116 OD of fermented media. The organism produced 0.807 OD confirming the validity.

Figure 2: Interaction between various factors on biomass production

a. Effect of pH and sea water

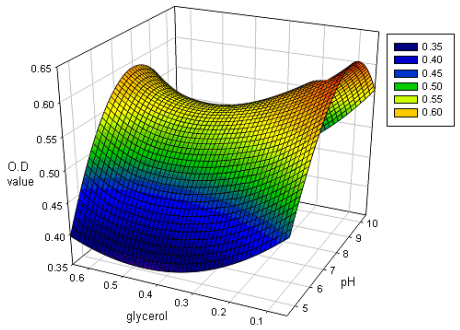


b. Effect of pH and sucrose

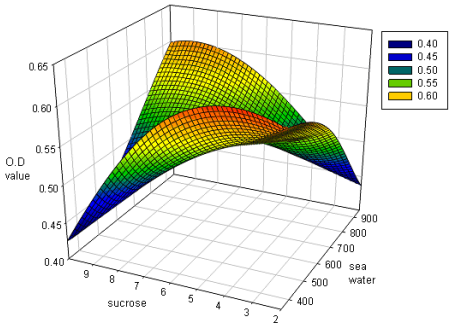


c. Effect of pH and glycerol

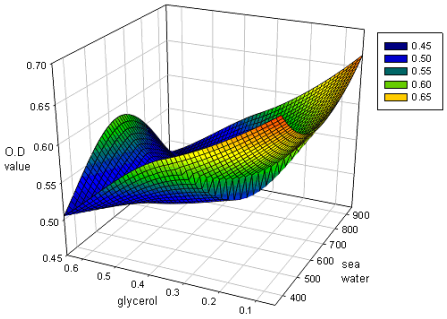
d. Effect of sea water and sucrose

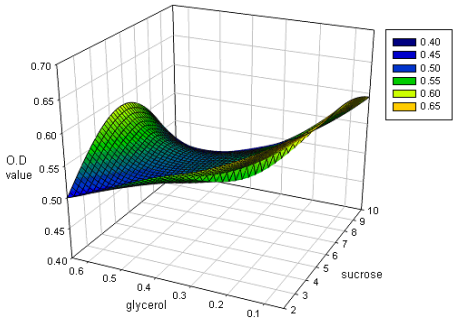


e. Effect of sea water and glycerol



f. Effect of sucrose and glycerol





[IV] CONCLUSION

The pigment production of the organism *Exiguobacterium* sp. was directly proportional to the growth of the organism and hence the yield of biomass was optimized using Plackett-Burman and Central Composite Design. The combination of PB design with CCD design for optimizing the bioprocess variables for biomass production by *Exiguobacterium* sp. is an effective and reliable tool to select the statistically significant factors and finding the optimal concentration of those factors in culture medium. Out of eight factors selected pH, seawater, sucrose, glycerol were found to have positive influence on biomass production. From CCD, the most optimum condition for biomass production was found to be pH 8.7, 350 mL/L of sea water, 2.0 g/L of sucrose and 0.5 mL/L glycerol. In addition, validation of the model suggested, unequivocally, the reliability of RSM for optimization of media for biomass production.

ACKNOWLEDGEMENT

The authors are thankful to Bharathiar University, Coimbatore, Tamil Nadu, India for providing facilities to undertake this study.

REFERENCES

1. Akhnazarova S, Kafarov V. (1982) *Experiment optimization in chemistry and chemical engineering*, Mir publications, Moscow and Chicago pp.245-262..

2. Anand TP, Bhat AW, Shouche YS, Roy U, Siddharth J, Sarma SP. (2006) Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East. India. *Microbiol. Res.* 161(3): 252-262.

3. Carte BK. (1993) Marine natural products as a source of novel pharmacological agents. *Curr. Opin. Biotechnol.* 4:275-279.

4. Cetina A, Matos A, Garma G, Barba H, Vázquez R, Rodríguez AZ, Jay D, Monteón V, Ruth López A. (2010) Antimicrobial activity of marine bacteria isolated from Gulf of Mexico. *Rev. peru. biol.* 17(2): 231 - 236

5. Deepak V, Kalishwaralal K, Ramkumarpandian S, Venkatesh Babu S, Senthilkumar SR, Sangiliyandi G. (2008) Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. *Bioresource Technology* 99(17): 8170-8174.

6. Dhanya G, Swetha S, Madhavan NK, Rajeev S, Ashok P (2008). Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. *Bioresource Technology* 99(11): 4597-4602.

7. Jayaswal RK, Fernandez MA, Schroeder RG. (1990). Isolation and characterization of a *Pseudomonas* strain that restricts growth of various phytopathogenic fungi. *Appl. Environ.Microbiol*. 56: 1053–1058

8. Jensen PR, Fenical W. (1994) Strategies for the Discovery of Secondary Metabolites from Marine Bacteria: Ecological Perspectives. *Annual Review of Microbiology* 48: 559-584

9. Khuri AI, Cornell JA. (1987) *Response Surfaces: Designs and Analyses*. Marcel Dekker, Inc., NewYork.

10. Lee SY, Jong HC, Zhaohui X. (2003) Microbial cell-surface display. *Trends Biotechnol.* 21: 45–52.

11. Lemos ML, Toranzo AE, Barja JL. (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microbiol. Ecol.* 11: 149-163.

12. Plackett RL, Burman, JP. (1946) The Design of Optimum Multifactorial Experiments. *Biometrika* 33(4):305-325.

13. Reichenbach H. (2001) Myxobacteria, producers of novel bioactive substances. *J Ind Microbiol Biotechnol* 27(3): 149–156.

14. Schneider G, Rheinheimer J. (1988) Isolation methods. In B. Austin (Ed.), *Methods in aquatic bacteriology.* pp. 73–94. New York, NY: Wiley.

15. Soliman NA, Berekaa M, Abdel-Fattah YR. (2005) Polyglutamic Acid (PGA) production by *Bacillus* sp. SAB-26: Application of Plackett-Burman experimental design to evaluate culture requirements. *Applied Microbiol. Biotechnol.* 69: 259-267.

16. Sreekumar G, Krishnan S. (2010) Enhanced biomass production study on probiotic *Bacillus subtilis* SK09 by medium optimization using response surface methodology. *African Journal of Biotechnology* 9 (47):8078-8084.

17. Uzair B, Ahmed N, Ahmad VU, Mohammad FV, Edwards DH. (2008) The isolation, purification and biological activity of a novel antibacterial compound produced by *Pseudomonas stutzeri. FEMS Microbiol. Lett.* 279:243-240.

18. Xiong ZQ, Tu XR, Tu GQ. (2008) Optimization of medium composition for actinomycin X2 production by *Streptomyces* spp. JAU4234 using response surface methodology. *J. Ind. Microbiol. Biotechnol.* 35:729–734.

19. Yee L, Blanch HW. (1993) Defined media optimization for the growth of recombinant *Escherichia coli* x90. *Biotechnol. Bioeng* 41:221-227.