Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 7, 2014

Original Article

OPTIMIZATION OF MEDIUM COMPONENTS FOR ANTIBACTERIAL METABOLITE PRODUCTION FROM MARINE *STREPTOMYCES* SP. PUA2 USING RESPONSE SURFACE METHODOLOGY

P. SHEKAR[1], K. SATHISH KUMAR[1], S. ANURADHA JABASINGH[2], M. RADHAKRISHNAN[3], R. BALAGURUNATHAN[4,*]

[1]Department of Chemical Engineering, SSN College of Engineering, Chennai 600110, Tamil Nadu, India, [2]Departmnet of Chemical Engineering, Sathyabama University, Chennai 600119, Tamil Nadu, India, [3]Centre for Drug Discovery and Development, Sathyabama University, Chennai 600119, Tamil Nadu, India, [4]Department of Microbiology, Periyar University, Salem 636011, Tamil Nadu, India
Email: rbalaguru@yahoo.com

Received: 10 May 2014 Revised and Accepted: 20 June 2014

ABSTRACT

Objective: The present study is an attempt to optimize the fermentation conditions for the antibacterial compound production from a newly isolated marine *Streptomyces* strain PUA2 by adopting response surface methodology as the statistical tool.

Methods: Prior to using the Response Surface Methodology, Plackett Burmann (PB) design was used to explore the effect of variables on the antibacterial compound production. In PB method, high and low values were assigned for the eight variables *viz.,* glucose, glycerol, soybean meal, manganese chloride, calcium carbonate, peptone and pH. Calcium carbonate and peptone were used as dummy variables. Based on the results of combined effects glycerol, soybean meal, manganese chloride and pH were investigated by 2^4 full-factorial central composite design.

Results: The results of PB method showed the significant effect of glycerol, soybean meal, manganese chloride and pH on the antibacterial compound production. The results of ANOVA and regression of second order model showed that the linear effects of glycerol and manganese chloride and cross products effects of manganese chloride and pH were more significant. All the critical variables having greatest effect on the production of antibacterial compound from marine *Streptomyces* species PUA2. Optimization of process parameters resulted in increase in antibacterial activity from 7 mm to 14 mm.

Conclusion: The factors optimized in the present study were useful for the increased production of antibacterial metabolite from *Streptomyces* sp PUA2. The result confirms the feasibility of medium optimization to improve antibiotic production.

Keywords: Antibacterial activity, Marine actinobacteria, Central Composite Design, Plackett Burmann design, Response surface methodology.

INTRODUCTION

Marine actinobacteria are metabolically active and physiologically adoptive to growth in sea water. They are the promising source for novel secondary metabolites with different biological activities like antimicrobial, anticancer, antiviral, anti-inflammatory and antiparasitic, etc. Even with the limited screening efforts dedicated to date to marine actinobacteria, the discovery rate of novel secondary metabolites from marine actinobacteria has recently surpassed that of their terrestrial counterparts, as evident by the isolation of many new chemical entities from marine actinobacteria [1-2]. During the period of 2003-2005, years, 659 marine bacterial compounds have been described in which 256 compounds have originated from actinobacteria [3].

Bioactive metabolites from actinobacteria are commonly produced by submerged or solid state fermentation with one or two commonly used media. Optimizing the suitable conditions and medium components is a prerequisite for better production of antibiotics or any other metabolites. Medium optimization explores a sequence of phases with a specific set of optimal conditions fixed by different methodologies.

Nutritional aspects like carbon source, nitrogen source and minerals and other conditions like time of incubation, pH and temperature are known to have profound effect on the production of antimicrobial metabolites by actinobacteria. Hence optimization of culture conditions is very essential to get maximum yield of antimicrobial metabolites [4-6].The optimization of the medium by the conventional research technique, the classical one factor at a time method involves changing one independent variable like carbon source and nitrogen source, while fixing others at constant levels. Optimal medium is not always obtainable by the traditional one factor at a time optimization strategy because of potential interactions among medium components [7]. PB design is a well known and widely used statistical technique for screening and selection of most significant culture variables. PB design offers a good and fast screening procedure and mathematically it computes the significance of a large number of factor variables in one experiment, which saves time and maintains convincing information on each component [8-9]. This method allows the screening of main factors from a large number of experiments that can be retained in further optimization processes.

RSM is a powerful technique for testing multiple process variables owing to fewer experimental trials that are needed compared to the study of one variable at a time. Also, significant interactions between the variables can be identified and quantified by this technique. RSM has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters. A prior knowledge and understanding of these parameters are necessary for achieving a more realistic model [10]. The present study reports the effect of critical medium components on antibacterial metabolite production from a marine *Streptomyces* sp PUA2 using Placket Burmann design and Response Surface Methodology (RSM).

MATERIALS AND METHODS

Streptomyces sp PUA2 strain

Streptomyces sp. PUA2 was isolated from marine sediment collected from Andaman ecosystems (Lat. 11.68°N;Long 92.77°E) using starch casein agar medium. It produced dirty white powdery colonies with diffusible pigment production on ISP2 agar medium. Viability of strain PUA2 was maintained on ISP2 agar slants at 4°C. Crude ethyl acetate extract prepared from *Streptomyces sp* PUA2 showed promising activity against the gram positive and gram negative bacterial pathogens. In an earlier study, effect of critical medium components on bioactive metabolite production from *Streptomyces* sp PUA2 was determined by classical one factor at a time method [11].

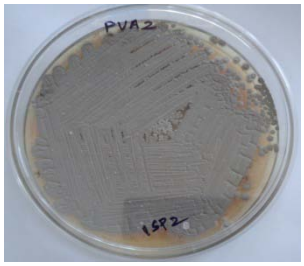

Fig. 1: Growth of *Streptomyces* sp PUA2 on ISP2 agar

Inoculum preparation

Well grown culture of *Streptomyces* sp PUA2 was transferred in to 100 ml yeast extract malt extract (YEME) and kept in rotary shaker for 48 h. Basal medium consists of 1% glucose;1% yeast extract and 0.1% NaCl. The pH of the medium was adjusted to 7.0 prior to sterilization. Each 25 ml of basal medium was prepared and inoculated with different variables in respective concentrations.

Experimental procedure

Antibacterial activity of cell free supernatant was tested by agar well diffusion method. Previously cultured *Staphylococcus aureus* MTCC96 was inoculated on nutrient agar plates using sterile cotton swab. A 5 mm diameter well was made on the nutrient agar plates and loaded with 50 µl cell free supernatant. The inoculated plates were incubated at 37°C and were kept under observation for 24 hours for determining the zone of inhibition. Dry weight of biomass was estimated using preweighed tube and expressed in mg of biomass per ml of the medium.

Plackett Burmann Screening Design (PB)

PB design was used for screening the most significant factors affecting the production of antibacterial metabolites. Each independent variable was tested at high and low levels indicated by + and-respectively. Effect of variables on bioactive metabolite production was determined by applying a two level factorial design. Different combinations and sequence of process conditions were investigated to determine the growth conditions best suited for growth and biomass production [12]. High and low values were assigned for the selected variables. Plackett Burmann designs for the seven variables were assigned as X_1, X_2, X_3, X_4, X_5, X_6, and X_7 at high and low levels in which two factors, X_4 and X_6 were designated as 'dummy' variables. The factors or independent variables considered for design include glucose (X_1), glycerol (X_2), soybean meal (X_3), calcium carbonate(X_4), manganese chloride (X_5), peptone (X_6) and pH (X_7) respectively. The results obtained with classical experiments in the previous study [11] were used in the selection of independent and dummy variables.

Table 1: Medium components and test levels for plackett-burmann experiment

Variables	Medium components	Low Level (-)	High Level (+)
X_1	Glucose	0.5	1.5
X_2	Glycerol	0.5	1.5
X_3	Manganese chloride	0.05	0.15
X_4	Calcium carbonate	0.5	0.5
X_5	Soybean Meal	0.05	0.15
X_6	Peptone	0.5	0.5
X_7	pH	6	8

Response surface methodology using central composite design (CCD)

Central composite design is one of the response surface methodologies. For statistical calculations the variables X_i were coded as x_i according to Equation 1.

$Xi = (x_i - x_0)/\Delta x_i$, (i=1, 2, 3...k) ..... (1)

Where X_i is (dimensionless) coded value of the real variable x_i, x_0 is the realvalue of X_i at the center point (zero) level, and the Δx_I is the step change value. Each variable in the design were coded at five levels-2,-1, 0, 1, and 2 with all variables taken at a central coded value of zero. Accordingly, a 2^4 factorial experiment design, with an axial point (10mm) and six replicated at the center point, with a total number of 30 experiments were employed. Second degree polynomials, which include all interaction term, were used to calculate the predicted response in equation 2.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{..... (2)}$$

Where Y represents response variables, β_0 is the interception coefficient, βi coefficient of the linear effect, β_{ii}, the coefficient of quadratic effect and βij, the coefficient of interaction effect.

RESULT AND DISCUSSION

Optimization by Plackett Burmann method

Plackett-Burmann statistical design was applied to reflect the relative importance of various fermentation factors. Seven different variables including fermentation conditions and medium constitution were chose to perform this optimization process. Antibacterial activity was measured after 120 hours of incubation [13]. The design of independent variables coded with the corresponding medium composition values with high level (H) and low level (L). The high level of each variable was set far enough from the low level to identify which ingredients of the media have significant influence on the antibacterial metabolite production [14]. The main effect of each variable on antibacterial activity was estimated as the difference between both averages of measurements made at the high level and at the low level of that factor. The data given in Table 2 showed a wide variation from 0 to 8 mm of inhibition zone. The variation reflects the importance of medium optimization to attain higher productivity. The main effects of the examined factors on the antimicrobial activity were calculated shown in Table 3. According to the resulting effects of 7 variables on antibacterial activity, glycerol (X_1),

Effect of sources and their interaction on biomass

For each trial carried out experimentally by the Plackett Burmann design for the different combination of high and low values, biomass were determined. Trial 2 recorded highest biomass weight (17.7 mg/mL) while trial 8 has lowest biomass weight (6 mg/mL) [17-18]. The values were shown in Table 2 and data were analyzed graphically in figure 2.

Optimization by Response Surface Methodology

The optimum concentrations of the individual components in the production medium for antibacterial compound production by *Streptomyces* sp PUA2 were further optimized by the Response Surface Methodology. According to this design, the total number of treatment combinations was 2^k+2k+n_o where k is the number of independent variables and n_0 is number of repetitions of experiment at centre point. Based on the results of PB design, four critical components of the production medium were selected. For RSM analysis based on CCD, 30 experiments were performed in triplicate. The coded levels of the independent variables are given in

Table 4. A 2^4 factorial CCD was developed by design expert package version 9.0.2 with 8 axial points and 6 replicates at the center points leading to 30 runs [19]. Multiple regression analysis was applied on the experimental data by the CCD design. The Results were fitted with a second order full polynomial equation. The empirical relationship between antibacterial metabolite production and the four test variables n coded units obtained the application of RSM is given by below equation.

Table 2: Plackett burmann experimental design for evaluating factors influencing antibacterial metabolite production

Trial No	Variables							Antibacterial activity against *S. aureus* MTCC96(mm)	Biomass(mg/mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7		
1	H	H	H	L	H	L	L	6.5	8.3
2	L	H	H	H	L	H	L	6	17.7
3	L	L	H	H	H	L	H	0	8.4
4	H	L	L	H	H	H	L	8	8.4
5	L	H	L	L	H	H	H	7	13.7
6	H	L	H	L	L	H	H	0	10
7	H	H	L	H	L	L	H	6	8.2
8	L	L	L	L	L	L	L	4	6

Table 3: Statistical calculations for plackett burmann method

	X_1	X_2	X_3	X_4	X_5	X_6	X_7
Sum of [H]	20.5	25.5	12.5	20	21.5	21	13
Sum of [L]	17	12	25	17.5	16	16.5	24.5
Effect	0.875	3.38	-3.13	0.63	1.38	1.13	-2.88
MS	1.531	22.78	19.53	0.78	3.78	2.53	16.53
F-test	0.92	13.80	11.83	0.472	2.29	1.53	10.01

MS-mean square;EMS-error mean square = 1.65, soybean meal (X_3), pH (X_7) and manganese chloride (X_5) have significantly influenced antibacterial metabolite production and the dummy variables (X_4 & X_6) and glucose (X_1) included in the design have a least effect on the antibacterial compound production based on F-test values. The mean square (variance of effect) showed higher value for glycerol. The highest antibacterial activity was recorded in trial 4 while the lowest antibacterial activity was in Trial 8 and no activity in Trial 5 and 6 [15-16].

Table 4: Range and levels of different process variables used in ccd for antibacterial activity

Variables	-2	-1	0	1	2
Glycerol(X_1) g/mL	5	7.5	10	12.5	15
Soybean meal (X_2) g/mL	5	7.5	10	12.5	15
Manganese chloride (X_3) g/mL	0.5	0.75	1	1.25	1.5
pH(X4) 1-9	5	6	7	8	9

$$Y = 12.17 + 0.083X_1 + 4.53\times10^{-17}X_2 + 0.41X_3 + 0.33X_4 - 1.82\times10^{-16}X_1X_2 - 2.29\times10^{-16}X_1X_3 - 3.94\times10^{-16}X_1X_4 + 0.13X_2X_3 - 0.12X_2X_4 + 0.13X_3X_4 - 1.08X_1^2 - 1.08X_2^2 - 0.96X_3^2 - 0.58X_4^2$$

Where Y is the measured response (antibacterial activity units in mm), X_1, X_2, X_3 and X_4 are the coded values of independent variables. CCD results on experiments with different combination for X_1, X_2, X_3, and X_4 are presented along with predicted and experimental values for antibacterial activity shown in table 5.

The coefficient of determination (R^2) was found to be 0.755 for the antibacterial metabolite production, indicating 75.5 % of variability in the response could be explained by the statistical model. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. In this case, the value of the determination of coefficient elucidated the validity of the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model.

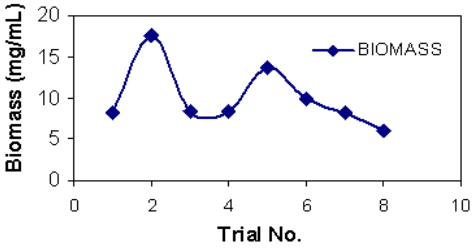

Fig. 2: Showing Biomass (mg/ml) vs. Trial no in Plackett Burmann Method.

The value of the adjusted determination coefficient (Adj R^2 = 0.53) was also high supporting the significance of the model [20]. In this case the adjusted R^2 was noticeably smaller than the R^2 value since there are many variables in the model and their sample size was also not very large [21]. The coefficient of variation of model was 4.22% and the PRESS statistic value of 118.32 proved the validity of model.

There is a 38.03% chance that a lack of fit has occurred and this could be due to noise. Non-significant lack of fit is good for an apt model showing excellent optimization [22-23]. Response surface contour plots and their three dimensional graphs are employed to understand the relationship between the response and experimental values of each variable. These plots also showed the type of interaction between test variables and helped to obtain the optimum conditions [24]. A total of six response surface plots were obtained by the central composite design. Maximum zone of inhibition showing the antibacterial activity (12.09 mm) was found during the interaction of soybean meal and Glycerol by fixing the quantity of manganese chloride and pH (Fig.1a). The antibacterial activity was observed in terms of the zone of inhibition, 10.78 mm as shown in

the Fig.1b at pH 7 and glycerol (10g/mL). Similar values of the inhibition zone were observed during the interaction of Manganese chloride (1g/mL) and glycerol (10g/ml). The zone of inhibition was 10.67 mm during this interaction (Fig.1c). A comparable inhibition zone of 12.01 mm (Fig.1d) was obtained at pH 7 and soybean meal (10g/ml), The interaction of Manganese chloride (1gm/ml) and Soybean meal(10g/ml) showed an intermediate zone of inhibition, whose value was observed as 11mm (Fig 1e). Figure.1f shows the interaction between manganese chloride (1g/ml) and pH 7 and this interaction produced an inhibition zone of 11.5 mm [25-27].

Table 5: Central composite design matrix in coded values and responses

	Variables				Antibacterial activity against *S.aureus MTCC96*(mm)	
Run	**X_1**	**X_2**	**X_3**	**X_4**	**Experimental value**	**Predicted value**
1	-1	-1	-1	-1	7	7.726
2	1	-1	-1	-1	7	8.117
3	-1	1	-1	-1	7	8.547
4	1	1	-1	-1	7	10.683
5	-1	-1	1	-1	7	10.131
6	1	-1	1	-1	8	11.762
7	-1	1	1	-1	8	11.102
8	1	1	1	-1	8	7.279
9	-1	-1	-1	1	8	7.534
10	1	-1	-1	1	8	7.359
11	-1	1	-1	1	8	7.415
12	1	1	-1	1	8	11.943
13	-1	-1	1	1	9	11.247
14	1	-1	1	1	9	12.082
15	-1	1	1	1	9	11.187
16	1	1	1	1	9	9.195
17	-2	0	0	0	9	8.70
18	2	0	0	0	9	8.557
19	0	2	0	0	9	9.517
20	0	-2	0	0	9	7.141
21	0	2	0	0	9	14.269
22	0	0	2	0	10	9.141
23	0	0	-2	0	11	9.269
24	0	0	0	-2	11	8.308
25	0	0	0	2	11	8.308
26	0	0	0	0	11	8.308
27	0	0	0	0	12	8.308
28	0	0	0	0	12	8.308
29	0	0	0	0	13	8.308
30	0	0	0	0	14	8.308

Table 6: Analysis of variance (anova), regression coefficient estimate and test of significance for antibacterial activity in mm (r1)

Source	**Sum of Squares**	**Df**	**F Value**	**P Value Prob > F**
Model	79.13	14	3.30	0.0141
X_1	0.17	1	0.097	0.7593
X_2	2.842×10^{-14}	1	1.661×10^{-14}	1.0000
X_3	4.17	1	2.44	0.1395
X_4	2.67	1	1.56	0.2310
X_1^2	32.19	1	18.81	0.0006
X_2^2	32.19	1	18.81	0.0006
X_3^2	25.19	1	14.72	0.0016
X_4^2	9.33	1	5.45	0.0338
X_1X_2	4.263×10^{-14}	1	2.492×10^{-14}	1.0000
X_1X_3	2.842×10^{-14}	1	1.661×10^{-14}	1.0000
X_1X_4	2.842×10^{-14}	1	1.661×10^{-14}	1.0000
X_2X_3	0.25	1	0.15	0.7076
X_2X_4	0.25	1	0.15	0.7076
X_3X_4	0.25	1	0.15	0.7076
Residual	25.67	15		
Lack of Fit	18.83	10	1.38	0.3803
Pure Error	6.83	5		
Correlation Total	104.80	29		

Statistical significance of model was investigated by ANOVA as shown in Table 6. The F value of 3.30 implies the significance of the model. P values of the model (0.0141) indicate the significance of the model terms. In this case X_1^2, X_2^2, X_3^2 and X_4^2 are significant model terms. The p value of the Lack of fit term was 1.38 implying its non-significant relative to pure error.

In Response plots and contour diagrams (Fig.3), contour diagrams (Fig.1a,1d, 1e, 1f) were elliptical and tilted, indicating significant cross product interaction between the factors X_1 and X_2, X_2 and X_3, X_2 and X_4, X_3 and X_4 [28-29].This is also evident from coefficient

estimate values (Table 6). Contour diagrams of Figure 1b and Figure 1d are elliptical but not tilted, indicating a good deal of negligible interaction between (X_1 & X_3 and X_1 & X_4). This implies that the linear effects of glycerol, manganese chloride and pH are significant. The interactive effect of soya bean meal with manganese chloride and pH is more significant than the other factors. These suggest that the concentrations of glycerol, manganese chloride and pH have a direct relationship with the production of antibiotic and creation of the zone of inhibition In the present study, the experimental results based on response surface approach clearly showed that the antibiotic production was dependent on the optimized values of the medium components taken into consideration [30-32].

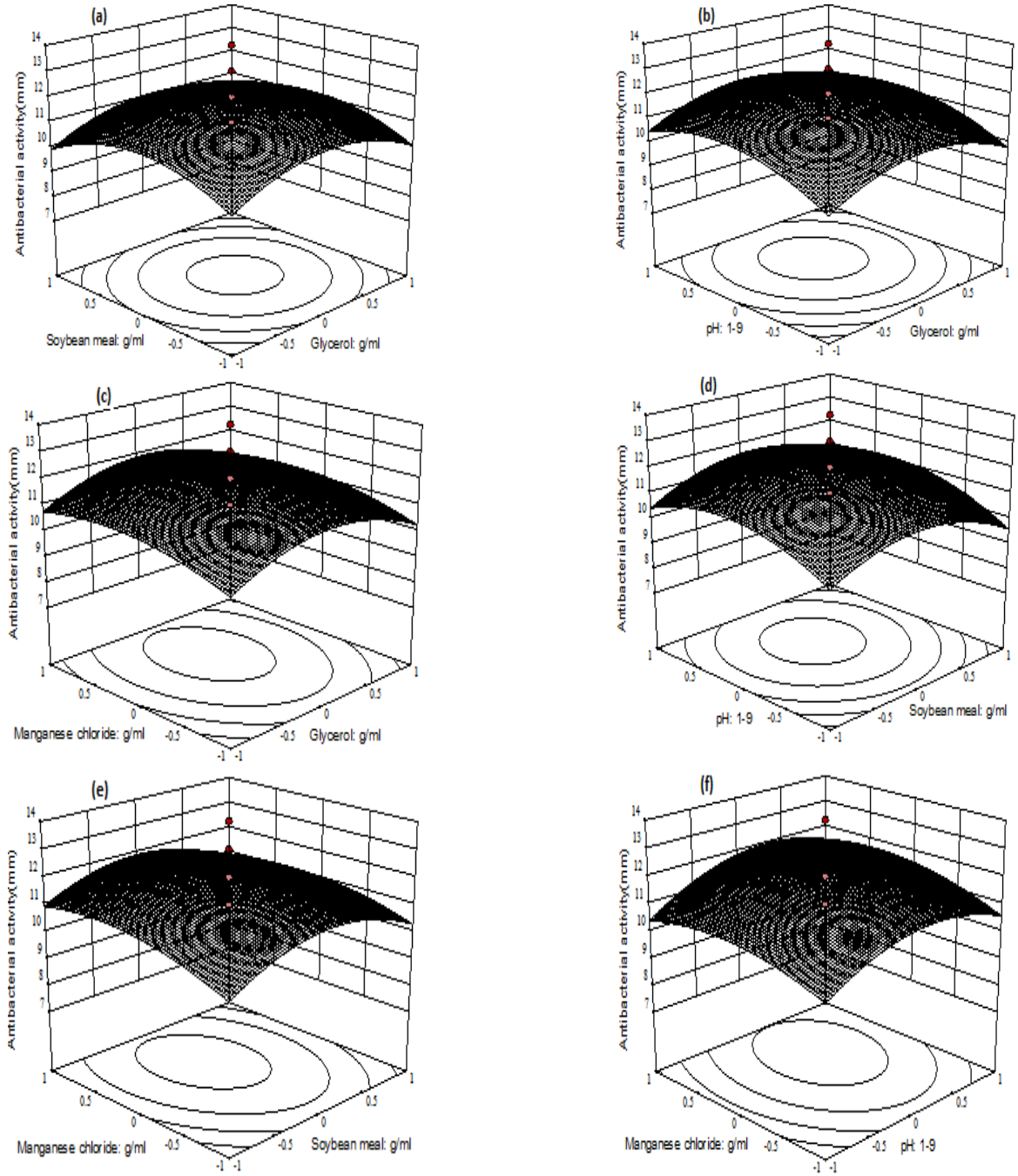

Fig. 3: Response surface plots for Antibacterial activity:a) Soy bean meal vs. glycerol;b) pH vs. Glycerol;c) Manganese chloride vs. Glycerol;d) pH vs. Soy bean meal;e) Manganese chloride vs. Soybean meal;f) Manganese Chloride vs. pH

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGEMENT

Authors thank the authorities of Periyar University for the research facilities provided.

REFERENCES

1. Lam KS. Discovery of novel metabolite from marine actinomycetes, Current Opinion in Microbiology 2006;9:245-51.
2. Radhakrishnan M, Balagurunathan R, Selva Kumar N, Doble M, Vanaja Kumar. Bioprospecting of marine derived actinomycetes with special reference to antimycobacterial activity, Indian J Geo-Marine Sci 2011;40(3):407-10.

3. Williams, P.G, Panning the chemical gold:marine bacteria as a source of new therapeutics. Trends Biotechnol 2009;27:45-52.
4. Ripa FA, Nikkon F, Zaman S, Khondkar P. Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp.RUPA-08PR isolated from Bangladesh soil. Mycobiology 2009;37(3):211-14.
5. Kiranmayi MU, Sudhakar P, Sreenivasulu K, Vijayalakshmi M. Optimization of culturing conditions for improved production of bioactive metabolites by *Pseudonocardia* sp. VUK-10. Mycobiology 2011;39(3):174-81.
6. Song Q, Huang Y, Yang H. Optimization of fermentation conditions for antibiotic production by actinomycetes YJ1 strain against *Sclerotinia sclerotiorum*. J Agri Sci 2012;4(7):95-102.
7. Prakasham RS, Subba Rao R, Sreenivas Rao R, Rajesham S, Sarma PN. Optimization of alkaline protease production by *Bacillus* sp. using Taguchi methodology. Applied Biochemistry and Biotechnology 2005;120:133-44.
8. Conley W. Computer Optimization Techniques. Revised ed. Petrocelli Books:Princeton NJ, 1984;147-63.
9. Yasser R, Abdel-Fattah, Saeed HM, Gohar YM, El-Baz MA. Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochemistry 2005;40(5):1707-714.
10. Box GEP, Hunter WG, Hunter JS, Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building, New York:John Wiley and Sons, 1978.
11. Shekar P, Anuradha J, Radhakrishnan M, Balagurunathan R. Effect of critical medium components on antimicrobial compound production from marine *Streptomyces* species(A2) by one factor at a time method. J Chem Pharm 2011;3(6):872-76.
12. Bashir ZM, Ahmad A, Nor SM, Usup G. Factors affecting bioactivity of secondary metabolites produced by *Streptomyces* sp. PT1 using Plackett-Burman design. Advance in Environmental Biology 2012;6(12):3043-51.
13. Vijayakumar R, Paneerselvam K, Muthukumar C, Thajuddin N, Paneerselvam A, Saravanamuthu R. Optimization of antimicrobial production by a marine actinomycete *Streptomyces afghaniensis* VPTS3-1 isolated from Palk Strait, East Coast of India. Indian J Microbiol 2012;52(2):230-39.
14. Jian Z, Fa GN. Application of response surface methodology in medium optimization for pyruvic acid production of *Torulopsis glabrata* TP19 in batch fermentation. Journal of Zhejiang University SCIENCE B 2007;8(2):98-104.
15. Sharma D, Manhas RK. Application of Plackett–Burman experimental design and Box and Wilson design to improve broad-spectrum antimicrobial compound. Indian Journal of Biotechnology 2013;12:386-94.
16. Anuradha Jabasingh S, Valli Nachiyar C. A new combinational statistical approach for cellulase optimization in *Aspergillus nidulans.* Indian Journal of Science and Technology, 2010;3(8):871-78.
17. El-Sersy NA, Abd-Elnaby H, Abou-Elela GM, Hibrahin HA, EI-Toukhy NMK. Optimization, economization and characterization of cellulase produced by marine *Streptomyces ruber*. African Journal of Biotechnology 2010;9(38):6355-364.
18. Liu GQ, Wang XL. Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei.* Appl Microbiol Biotechnol 2007;74:78-83.
19. Khuri AI, Cornell JA. Response Surfaces:designs and analysis, Marcel Decker, New York, NY, USA, 1987.
20. Himabindu M, Ravichandra P, Vishalakshi K, Jetty A. Optimization of critical medium components for the maximal production of Gentamicin by *Micromonospora echinospora* ATCC 15838 using Response Surface Methodology. Applied Biochemistry and Biotechnology 2006;134:143-53.
21. Anuradha Jabasingh S, Pavithra G. Response surface approach for the biosorption of Cr6+ ions by Mucor racemosus Clean—Soil Air Water 2010;38(5-6):492–99.
22. Shi LE, Qing G, Zhang XY, Tang ZX, Chen JS, Xiong WY, Liu HZ. Medium optimization for 5'-phosphodiesterase production from *Penicillium citrinum* using Response Surface Methodology. Food Technol Biotechnol 2007;45(2):126-33.
23. Jia B, Jin ZH, Mei LH. Medium optimization based on statistical methodologies for Pristinamycins production by *Streptomyces pristinaespiralis*. Appl Biochem Biotechnol 2008;144:133-43.
24. Haaland PD, Experimental design in Biotechnology, New York:Marcel Dekker;separating signals from the noise, 61–83;1989.
25. Cao Y, Meng DJ, Lu J, Long J. Statistical optimization of xylanase production by *Aspergillus niger* AN-13 under submerged fermentation using response surface methodology. African Journal of Biotechnology 2008;7(5):631-38.
26. Song S, Wei R, Zhang B, Wang D. optimization of the antibacterial activity of half-fin anchovy (setipinna taty) hydrolysates. Food Bioprocess Technol 2012;5:1979-989.
27. Amanda S, Motta, Brandelli A. Evaluation of environment conditions for productions of bacteriocin-like substance by *Bacillus* sp. strain P34. World J Microbiol Biotechnol 2008;24:641-46.
28. Anuradha Jabasingh S, Valli Nachiyar C. Optimization of cellulase synthesis by RSM and evaluation of ethanol production from enzymatically hydrolysed sugarcane bagasse using *Saccharomyces cerevisiae*. J Scientific and Industrial Res 2012;71:353-59.
29. Wang YH, Feng JT, Zhang Q, Zhang X. Optimization of fermentation condition for antibiotic production by *Xenorhabdus nematophila* with Response Surface Methodology. J Applied Microbiology 2008;104:735-44.
30. Anuradha Jabasingh S. Optimization and kinetics of cellulase immobilization on modified chitin using Response Surface Methodology. Adsorption Science & Technology 2011;29(9):897-915.
31. Anuradha Jabasingh S, Varma S, Pavithra G. Response Surface Methodology for the evaluation and comparison of cellulase production by *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC 344 cultivated on pretreated suguarcane bagasse. Chemical and Biochemical Engineering Quarterly 2014;28(1):127-35.
32. Anuradha Jabasingh S. Utilization of pretreated coir pith for the optimized bioproduction of cellulose by *Aspergillus nidulans.* International Biodeterioration & Biodegradation, 2011;65:1150-60.