International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 9 (6): 322-334, 2013
ISSN 1811-7775 / DOI: 10.3923/ijp.2013.322.334
© 2013 Asian Network for Scientific Information

Statistical Optimization of Process Variables for Antimicrobial Metabolites Production by *Streptomyces anulatus* NEAE-94 Against some Multidrug-resistant Strains

[1]Noura El-Ahmady El-Naggar, [2]Ashraf A. El-Bindary and [1]Noura Salah Nour
[1]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt
[2]Department of Chemistry, Faculty of Science, Damietta University, 34517, Damietta, Egypt

Abstract: *Streptomyces anulatus* NEAE-94, an antagonistic actinomycete newly isolated from Egyptian soil, exhibited a broad antimicrobial spectrum against several microorganisms including multidrug-resistant *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. Fifteen factors (starch, KNO_3, K_2HPO_4, yeast extract, NaCl, $MgSO_4$, $CaCO_3$, $FeSO_4$, pH, temperature, agitation speed, medium volume, inoculum size, inoculum age and fermentation time) were examined for their significances on production of antimicrobial metabolites using Plackett-Burman design. Among the variables screened, agitation speed, inoculum size and inoculum age had significant effects on antimicrobial activities production. These factors were further optimized using Box Behnken statical design.The optimal conditions achieved were high level of agitation speed (250 rpm min^{-1}), middle level of inoculum size (4%) and low level of inoculum age. The Antagonistic activity produced from the optimized culture conditions against multidrug-resistant *Staphylococcus aureus*, showed about two fold increase than that obtained from the un-optimized medium. As a result, a medium of the following formula was predicted to be the optimum for producing an extracellular antimicrobial metabolites in the culture filtrate of *Streptomyces anulatus* NEAE-94 L^{-1}: Starch 20 g, KNO_3 2 g, K_2HPO_4 0.5 g, NaCl 0.1 g, $MgSO_4.7H_2O$ 0.1 g, $CaCO_3$ 3 g, $FeSO_4.7H_2O$ 0.01 g, pH 6.5, temperature 25°C, agitation speed 250 rpm min^{-1}, medium volume 75 mL, inoculum size 4%,v/v, inoculum age 60 h and fermentation period 5 days.

Key words: *Streptomyces anulatus*, antimicrobial metabolites, Plackett-Burman design, Box-Behnken design, multidrug-resistant *Staphylococcus aureus*

INTRODUCTION

Antimicrobial drug resistance is one of the most serious problems because many bacteria that cause infections are becoming more resistant to commonly-used antibiotic treatments. *Staphylococcus aureus* bacteria is a common bacteria that is often on the skin and in the nose of healthy people. Staphylococci can cause a wide variety of diseases in humans either through toxin production or invasion (Muruganandham *et al*., 2010). *Staphylococcus* infections can be minor showing up as pimples, boils or skin infections or can be serious, leading to blood poisoning or lung infections. *Staphylococcus* infections may be due to cuts in the skin that b ecome infected, direct skin-to-skin contact or due to the ingestion of contaminated foods, typically animal products that are prepared on contaminated preparation surfaces or poorly refrigerated animal food products.

Staphylococci are becoming increasingly resistant to many commonly used antibiotics including penicillins, macrolides such as erythromycin, tetracyclines and aminoglycosides (DermNet, 2013). This is mainly due to the overuse of antibiotics and people not taking their antibiotic medicine properly. For example, the occurrence of methicillin-resistant *S. aureus* in hospitals has risen from less than 3% in the early 1980s to as much as 40% now (Sahin and Ugur, 2003). With the seemingly exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the need for discovering novel drugs is real (Sahin and Ugur, 2003; Dhanasekaran *et al*., 2009).

Corresponding Author: Noura El-Ahmady El-Naggar, Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt

Among bacteria, actinomycetes produce over two thirds of the clinically useful antibiotics of natural origin (Kieser *et al*., 2000), among which the major contributor was *Streptomyces*. The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades. Recent reports show that this group of microorganisms still remains an important source of antibiotics (Watve *et al*., 2001; Olano *et al*., 2008; Arasu *et al*., 2008). Most *Streptomyces* and other actinomycetes produce a diverse array of antibiotics including aminoglycosides (streptomycin and its relatives), anthracyclins, glycopeptides, β-lactams, macrolides (erythromycin and its relatives), nucleosides, peptides, polyenes, polyethers, tetracyclines, chloramphenicol, ivermectin, rifamycins and most other clinically-useful antibiotics (Goodfellow *et al*., 1988; Sahin and Ugur, 2003; Hotta and Okami, 1996; Baltz, 1998; Raja and Prabakarana, 2011).

The optimization of fermentation conditions, particularly physical and nutritional parameters, are of crucial importance to improve the efficiency of any fermentation process owing to their impact on the economy and practicability of the process, because it can significantly affect product concentration, yield and the ease and cost of downstream product separation (Wang *et al*., 2008). Medium optimization and physical conditions have been traditionally performed using one-factor-at-a time method. The disadvantages of such a classical method are that it is time consuming, laborious and expensive; in addition, it ignores the combined interactions among different variables (Jiang *et al*., 2008; Gao *et al*., 2009). Therefore, in recent years, numbers of statistical designs were used to search the key factors rapidly from a multivariable system, such as full factorial or Plackett-Burman design and response surface methodology (Gangadharan *et al*., 2008; Khanna and Srivastava, 2005; Wang and Lu, 2004; Ren *et al*., 2008; Aghaie-Khouzami *et al*., 2012). Response surface methodology has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production (Deepak *et al*., 2008; Liu and Wang, 2007; Sayyad *et al*., 2007). It can also be used to evaluate the relative significance of several variables simultaneously (Li *et al*., 2008).

A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing antimicrobial metabolites production under submerged fermentation by *Streptomyces anulatus* NEAE-94. The levels of the significant variables were further optimized using response surface methodology.

MATERIALS AND METHODS

Microorganisms and cultural conditions: *Streptomyces anulatus* NEAE-94 was kindly provided by Dr. Noura El-Ahmady El-Naggar (Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technologial Applications, Alexandria, Egypt). This isolate was maintained on slopes containing starch-nitrate agar medium (Waksman, 1959) of the following composition (g L^{-1}): Starch 20; KNO_3 2; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; $CaCO_3$ 3; $FeSO_4.7H_2O$ 0.01; agar 20 and distilled water up to 1L. Slopes were incubated for a period of 7 days at 30°C. The isolate was stored as spore suspensions in 20% (v/v) glycerol at -20°C (Hopwood *et al*., 1985) for subsequent investigation.

Antimicrobial agent activities were tested against a group of multidrug-resistant bacteria isolated from various clinical specimens and kindly provided by Infection Control Unit, Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt: *Staphylococcus aureus* A9897 (this strain is resistant to Vancomycin, Augmentin, Gentamicin, Trimethoprim-sulfamethoxazole, Oxacillin, Amikacin and Tobramycin), *Pseudomonas aeruginosa* T9934 (resistant to Ceftriaxone, Gentamicin,Cefotaxime, Trimethoprim-sulfamethoxazole and Augmentin) and *Klebsiella pneumonia* A9898 (resistant to Trimethoprim-sulfamethoxazole, Augmentin, Gentamicin, Ceftriaxone, Amikacin and Cefotaxime), The antimicrobial activities were also tested against a group of bacteria belonging to the Culture Collection of NRRL: Gram-positive (*Staphylococcus aureus* NRRL B-313, *Bacillus subtilis* NRRL B-543), Gram-negative (*Escherichia coli* NRRL B-210, *Pseudomonas aeruginosa* NRRL B-23) and *Candida albicans* NRRL Y-477. Stock cultures of the test organisms were maintained on Nutrient agar slants. The inoculated agar medium was incubated for 24 h at 30°C and then maintained at 4°C until further use.

Inoculum preparation: Two hundred and fifty milliliter Erlenmeyer flasks containing 50 mL of yeast-malt extract broth (malt extract 1%; dextrose 0.4%; yeast extract 0.4%; pH 7.0) were inoculated with three disks of 9 mm diameter (according to the method of Gill *et al*. (2003)) taken from the 7 days old stock culture grown starch nitrate agar medium. The flasks were incubated for 48-72 h in a rotatory incubator shaker at 30°C and 200 rpm and were used as inoculum for subsequent experiments.

Production conditions: Fifty milliliter or seventy five milliliter of fermentation medium were dispensed in 250 mL Erlenmeyer conical flasks, inoculated with previously prepared inoculum. The inoculated flasks were incubated on a rotatory incubator shaker at 150-250 rpm and 25-30°C. After the specified incubation time for each set of experimental trials, the mycelium of each isolate was collected by centrifugation at 5000 rpm for 15 min. The cell free supernatant was used for antimicrobial activities determinations.

Antagonistic action against microbial test strains: The well-diffusion technique was used to test the ability of the isolate to inhibit the growth of several Gram-positive, Gram-negative bacteria and *Canadida albicans*. Fifty milliliter of nutrient agar medium was poured into Petri plates. After solidifying, plates were inoculated with test strains and wells were punched out using 6 mm cork borer. One hundred microliter of tested filtrates was transferred into each well. All plates were incubated at 30°C for 24 h. After incubation period, the plates were observed for the inhibition zone formation around the wells. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter.

Selection of significant variables by Plackett-Burman design: The purpose of the first optimization step was to identify which ingredients of the medium have a significant effect on antimicrobial metabolites production. The Plackett-Burman statistical experimental design is a two factorial design, very useful for screening the most important factors with respect to their main effects (Krishnan *et al*., 1998; Yu *et al*., 1997). The total number of experiments to be carried out according to Plackett-Burman is n+1, where n is the number of variables (Plackett and Burman, 1946). Each variable is represented at two levels, high and low denoted by (+) and (-), respectively. Table 1 shows the fifteen different independent variables including starch, KNO_3, K_2HPO_4, yeast extract, NaCl, $MgSO_4$, $CaCO_3$, $FeSO_4$, pH, temperature, agitation speed, medium volume, inoculum size, inoculum age and fermentation time that chosen to be screened by Plackett Burman experiment. Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where, Y is the response variable (antimicrobial metabolites activity), β_0 is the model intercept and β_i is the linear coefficient and X_i is the level of the independent variable.

Table 1: Experimental independent variables at two levels used for the production of antimicrobial metabolites by *Streptomyces anulatus* NEAE-94 using Plackett-Burman design

Code	Independent variables	Levels -1	Levels +1
X_1	Starch (g L^{-1})	10	20.00
X_2	KNO_3 (g L^{-1})	1	2.00
X_3	K_2HPO_4 (g L^{-1})	0.5	1.00
X_4	Yeast extract (g L^{-1})	0	0.10
X_5	NaCl (g L^{-1})	0.1	0.50
X_6	$MgSO_4.7H_2O$ (g L^{-1})	0.1	0.50
X_7	$CaCO_3$ (g L^{-1})	1	3.00
X_8	$FeSO_4$ (g L^{-1})	0.01	0.02
X_9	pH	6.5	7.50
X_{10}	Temperature (°C)	25	30.00
X_{11}	Agitation speed (rpm min^{-1})	150	200.00
X_{12}	Medium volume (mL 250 mL^{-1} flask)	50	75.00
X_{13}	Inoculum size (%,v/v)	2	4.00
X_{14}	Inoculum age (h)	48	60.00
X_{15}	Fermentation time (day)	5	7.00

Table 2: Levels of variables chosen for the Box-Behnken optimization experiment

Variable	Variable code	-1	0	1
Agitation speed (rpm)	X_1	150	200	250
Inoculum size (%,v/v)	X_2	2	4	6
Inoculum age (h)	X_3	48	60	72

Response surface methodology (RSM): The levels and the interaction effects between various significant variables which exerted a positive effect on the antimicrobial metabolites production were analyzed and optimized by Box-Behnken methodology (Box and Behnken, 1960). In this study, the experimental plan consisted of 15 trials and the independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The levels of variables used for the study is shown in Table 2.

The experimental results of RSM were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_j \quad (2)$$

In which Y is the predicted response, β_0 is the regression coefficients, β_i is the linear coefficient, β_{ii} is the quadratic coefficients, β_{ij} is the interaction coefficients) and Xi is the coded levels of independent variables.

However, in this study, the independent variables were coded as X_1, X_2 and X_3. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}+ x_1x_3+\beta_{23}x_2x_3+\beta_{11}x^2{}_1+\beta_{22}x_2{}^2+\beta_{33}x^2{}_3 \quad (3)$$

Statistical analysis: The experimental data obtained was subjected to multiple linear regressions using Microsoft Excel 2007 to evaluate the analysis of variance (ANOVA) and to estimate the main effect, t-value, p-value and confidence level. The student t-test was used to determine the significance of the parameters regression coefficients. The p-values were used as a tool to check the significance of the interaction effects which in turn may indicate the patterns of the interactions among the variables (Montgomery, 1991). The quality of fit of regression model was expressed via the correlation coefficient (R), the coefficient of determination (R^2) and the adjusted R^2 and its statistical significance was determined by an F-test. Optimal value of activity was estimated using the solver function of Microsoft Excel tools. The statistical software package, STATISTICA software (Version 8.0, StatSoft Inc., Tulsa, USA) was used to plot the three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

RESULTS AND DISCUSSION

Antimicrobial activity: The antimicrobial activity of secondary metabolites was tested against Gram-negative, Gram-positive bacteria and *Candida albicans* (Table 3). The results showed that *Streptomyces anulatus* NEAE-94 exhibited interesting antimicrobial activities. There was a strong activity against *Staphylococcus aureus* NRRL B-313 and multidrug resistant *Staphylococcus aureus* A9897. Moreover, the halo diameter obtained with *Staphylococcus aureus* NRRL B-313, multidrug resistant *Staphylococcus aureus* and *Bacillus subtilis* NRRL B-543 were 27, 20, 20 mm respectively. While those obtained with *Escherichia coli* NRRL B-210, *Pseudomonas aeruginosa* NRRL B-23, multidrug resistant *Pseudomonas aeruginosa* T9934 and *Candida albicans* NRRL Y-477 were 18, 17, 15, 17 mm, respectively. There is no activity against multidrug resistant *Klebsiella pneumonia* A9898.

Screening of parameters using Plackett-Burman design: The experiment was conducted in 20 runs to study the effect of the selected variables. The design matrix selected for the screening of significant variables for antimicrobial metabolites production and the corresponding responses are shown in Table 4. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. All trials were performed in duplicate and the average of antimicrobial metabolites activities were treated as responses. *Streptomyces anulatus* NEAE-94 growth has been shown as small, yellow spherical pellets (Fig. 1)

Fig. 1: *Streptomyces anulatus* NEAE-94 growth in small, yellow spherical pellets form during the antimicrobial metabolites production in shake flasks after inoculation and incubation on a rotary shaker (200 rpm) at 30°C

Table 3: Antimicrobial activity of the antimicrobial metabolites produced by *Streptomyces anulatus* NEAE-94

Microorganisms	Specification	Inhibition zone diameter (mm)
Gram positive bacteria		
Bacillus subtilis	NRRL B-543	20
Staphylococcus aureus	NRRL B-313	27
Staphylococcus aureus A9897	Resistant to vancomycin, augmentin, gentamicin, trimethoprim-sulfamethoxazole, oxacillin, amikacin and tobramycin	20
Gram negative bacteria		
Pseudomonas aeruginosa	NRRL B-23	17
Pseudomonas aeruginosa T9934	Resistant to ceftriaxone, gentamicin,cefotaxime, trimethoprim-sulfamethoxazole and augmentin	15
Escherichia coli	NRRL B-210	18
Klebsiella pneumonia A9898	Resistant to trimethoprim-sulfamethoxazole, augmentin, gentamicin, ceftriaxone, amikacin and cefotaxime	Negative
Yeast		
Candida albicans	NRRL Y-477	17

Table 4: Plackett-Burman experimental design for evaluation of fifteen independent variables with coded values along with the observed antimicrobial metabolites activity

Trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Inhibition zone (mm)
1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	12.0
2	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	18.0
3	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	00.0
4	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	13.0
5	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	22.0
6	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	27.0
7	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	23.0
8	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	22.0
9	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	12.0
10	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	20.0
11	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	20.0
12	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	18.0
13	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	24.0
14	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	23.0
15	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	16.0
16	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	14.0
17	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	12.0
18	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	13.0
19	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	22.0
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	19.0

-1, low level; +1, high level

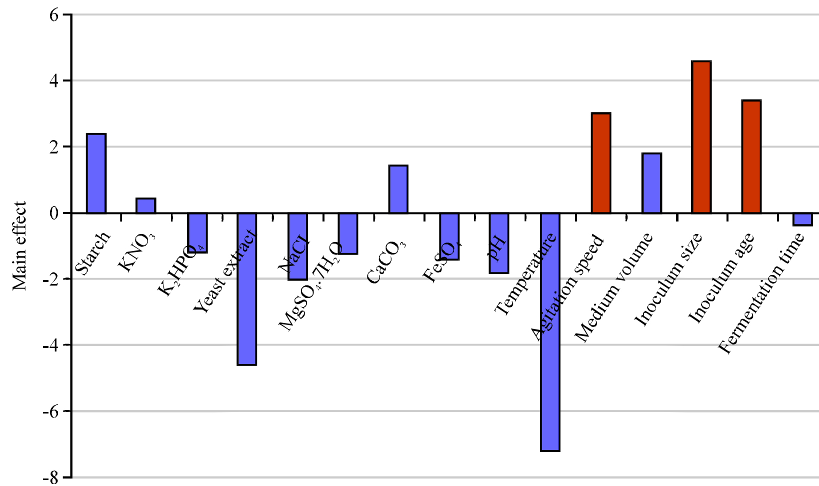

Fig. 2: Main effects of the fermentation conditions on antimicrobial metabolites production according to the Packett-Burman experimental results (the red color represent the most significant variables with positive effect)

production of the antimicrobial metabolites production in shake flasks. In submerged cultures, *Streptomyces* tends to form fluffy spherical pellets. Cell growth in the form of pellets led to better yield of antibiotic than growth as free filaments (Vecht-Lifshitz *et al*., 1989). Statistical analysis of the Plackett-Burman design and the responses were performed. The determination coefficient (R^2) values provide a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response (Kaushik *et al*., 2006). In this case, the value of the determination coefficient ($R^2 = 0.9576$) indicates that 95.76% of the variability in the response was attributed to the given independent variables and only 4.24% of the total variations are not explained by the independent variables. In addition, the value of the adjusted determination coefficient (Adj. $R^2 = 0.7984$) is also very high which indicates a high significance of the model (Akhnazarova and Kafarov, 1982). A higher value of the correlation coefficient ($R = 0.9785$) signifies an excellent correlation between the independent variables (Box *et al*., 1978) which indicates a good correlation between the experimental and predicted values. Thus, the analysis of the response trend using the model was considered to be reasonable.

With respect to the main effect of each variable (Fig. 2), we can see that seven variables from the fifteen named (starch, KNO_3, $CaCO_3$, agitation speed, medium

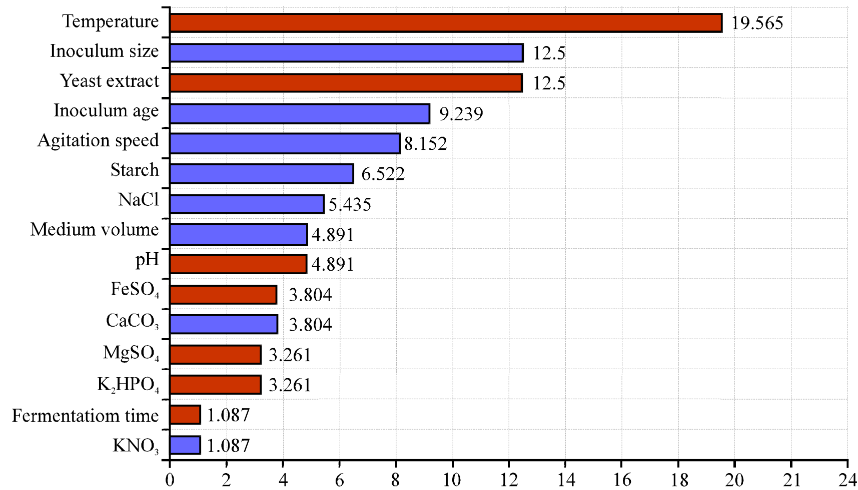

Fig. 3: Pareto chart illustrates the order of significance of the variables affecting the antimicrobial metabolites production by *Streptomyces anulatus* NEAE-94 (the red colour represent negative effects and the blue colour represent positive effects; Ranks (%) values ranging from 1.087 to 19.565)

Table 5: Analysis of variance (ANOVA) for optimization of antimicrobial metabolites production using Plackett-Burman design.

Variables	df	SS	MS	F	Significance F
Regression	15	690.40	46.0266	6.0166	0.0477
Residual	4	30.60	7.65		
Total	19	721.00			

df: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares, F: Fishers's function, Significance F: Corresponding level of significance

volume, inoculum size and inoculum age) were positively affect antimicrobial metabolites production, where the other eight variables named (K_2HPO_4, NaCl, $MgSO_4$, yeast extract, pH, temperature, $FeSO_4$ and fermentation time) negatively affect antimicrobial metabolites production. The variables with positive effect were fixed at high level. The variables which exerted a negative effect on antimicrobial metabolites production (the variables related to the growth medium composition and can't be eliminated) were maintained at their low (-1) level for further optimization by a response surface methodology while yeast extract with a negative effect will be excluded in the subsequent experiment. The Pareto chart illustrates the order of significance of the variables affecting antimicrobial metabolites production in Plackett-Burman Experimental Design (Fig. 3). Among the 15 variables, temperature showed the highest negative significance by 19.565%. Next to temperature, yeast extract showed higher negative effect by 12.5%. Inoculum size showed the highest positive effect by 12.5%.

The model F value of 6.0166 implies that the model is significant. The values of Significance F<0.05 (0.0477) indicate that model terms are significant (Table 5). The significance of each coefficient was determined by student's t-test and p-values which are listed in Table 6. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient (Akhnazarova and Kafarov, 1982). Some investigators have found that confidence levels greater than 70% are acceptable (Stowe and Mayer, 1966).Thus, in the current experiment, variables evidencing p-values of less than or equal to 0.075 (confidence levels exceeding 92.5%) were considered to have significant effects on the response. Temperature, with a probability value of 0.004, was determined to be the most significant factor, followed by yeast extract (0.020), inoculums size (0.020), inoculum age (0.051) and agitation speed (0.072). Screened significant variables, agitation speed, inoculum size and inoculum age exerted positive effects whereas the other variables, yeast extract and temperature exerted a negative effect on antimicrobial metabolites production. On the basis of the calculated t-values (Table 6), agitation speed (X_{11}), inoculum size (X_{13}) and inoculum age (X_{14}) were chosen for further optimization using Box-Behnken statistical design, since these factors had the most positive effects on the antimicrobial metabolites production.

Generally, suitable agitation speed lead to sufficient supply of dissolved oxygen in the media (Kumar and Takagi, 1999). Nutrient uptake by bacteria also will be increased (Beg *et al*., 2003).

Inoculum size can affect the metabolites accumulation. As the concentration of inoculum increases, it is followed by an increase in cell mass and after a certain period, metabolic waste interfere with the

Table 6: Statistical analysis of Plackett-Burman design showing coefficient values, t-test and p-values for each variable affecting antimicrobial metabolites production

Variables	Coefficients	Main effect	t-stat	p-value	Confidence level (%)
Intercept	17.5	35.0	28.296	0.000	99.999
Starch (g L^{-1})	1.2	2.4	1.940	0.124	87.566
KNO_3 (g L^{-1})	0.2	0.4	0.323	0.763	23.739
K_2HPO_4 (g L^{-1})	-0.6	-1.2	-0.970	0.387	61.309
Yeast extract (g L^{-1})	-2.3	-4.6	-3.719	0.020	97.951
NaCl (g L^{-1})	-1.0	-2.0	-1.617	0.181	81.879
$MgSO_4.7H_2O$ (g L^{-1})	-0.6	-1.2	-0.970	0.387	61.309
$CaCO_3$ (g L^{-1})	0.7	1.4	1.132	0.321	67.904
$FeSO_4$ (g L^{-1})	-0.7	-1.4	-1.132	0.321	67.904
pH	-0.9	-1.8	-1.455	0.219	78.069
Temperature (°C)	-3.6	-7.2	-5.821	0.004	99.566
Agitation speed (rpm)	1.5	3.0	2.425	0.072	92.766
Medium volume (mL 250 mL^{-1} flask)	0.9	1.8	1.455	0.219	78.069
Inoculum size (%, v/v)	2.3	4.6	3.719	0.020	97.951
Inoculum age (h)	1.7	3.4	2.749	0.051	94.856
Fermentation time (d)	-0.2	-0.4	-0.323	0.763	23.739

t: Student's test; p: Corresponding level of significance

production of metabolites due to which degradation of the product occurs. A lower inoculum density may reduce product formation, whereas a higher inoculum may lead to the poor product formation, especially the large accumulation of toxic substances and also cause the reduction of dissolved oxygen and nutrient depletion in the culture media (Mudgetti *et al*., 1986; Rahman *et al*., 2005). Low inoculum may require longer time for microbial multiplication and substrate utilization to produce desired product. On the other hand, high inoculum would ensure rapid proliferation of microbial biomass. So, balance between the proliferating biomass and substrate utilization would yield maximum enzyme activity as recorded by Ramachandran *et al.* (2004). Maximum antibiotic production was produced when 4% inoculum was used, further increase in the inoculum size did not have any significant increase on the production of bacitracin. It might be due to the reason that it consumed majority of the substrate for growth and metabolic processes, hence antibiotic synthesis decreased (Woolford, 1972). Adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing the growth of contaminants. Antibiotic production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis (Mahalaxmi *et al*., 2010). Abdel-Fatah (1996) found that the antifungal activity of *Streptomyces prunicolor* reached optimum level when inoculated the medium with 2% (v/v) of homogenized spore suspension of 5 days old culture. In addition, EL-Naggar *et al.* (2003) reported that maximum antibiotic production by *Streptomyces violatus* was obtained using inoculum size of 3 mL spore suspension 50 mL^{-1} liquid medium.

The quantity and quality of inoculum material play a crucial role in the bioprocess results. It was found that 72 h old inoculum at a size of 4% (v/v) gave best antibiotic production (Ettler, 1992). It has been found that 4% inoculums of the cells at stationary phase yielded the best growth and most consistent antibiotic production. Further increase or decrease in inoculum size reduced the antibiotic production. It could be due to the fact that cells of a younger inoculum were explained to be in a more active state in terms of multiplication, whereas an older inoculum could be partially or fully induced to product formation (Neves *et al*., 2000; Lopes *et al*., 2002).

By neglecting the terms that were insignificant ($p>0.072$), the first order polynomial equation was derived representing antimicrobial metabolites production as a function of the independent variables:

$$Y_{\text{(Antimicrobial metabolites production)}} = 17.5-2.3\,(X_4)-3.6\,(X_{10})+1.5\,(X_{11})+2.3\,(X_{13})+1.7\,(X_{14}) \quad (4)$$

where, Y is the response (antimicrobial metabolites production) and X_4, X_{10}, X_{11}, X_{13} and X_{14} are yeast extract, temperature, agitation speed, inoculum size and inoculum age; respectively. It can be seen from Eq. 4 that agitation speed, inoculum size and inoculum age exerted positive effect on antimicrobial metabolites production by *Streptomyces anulatus* NEAE-94.

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. Figure 4a shows the normal probability plot of the residuals, it is an important diagnostic tool to detect and explain the systematic departures from the normality. The

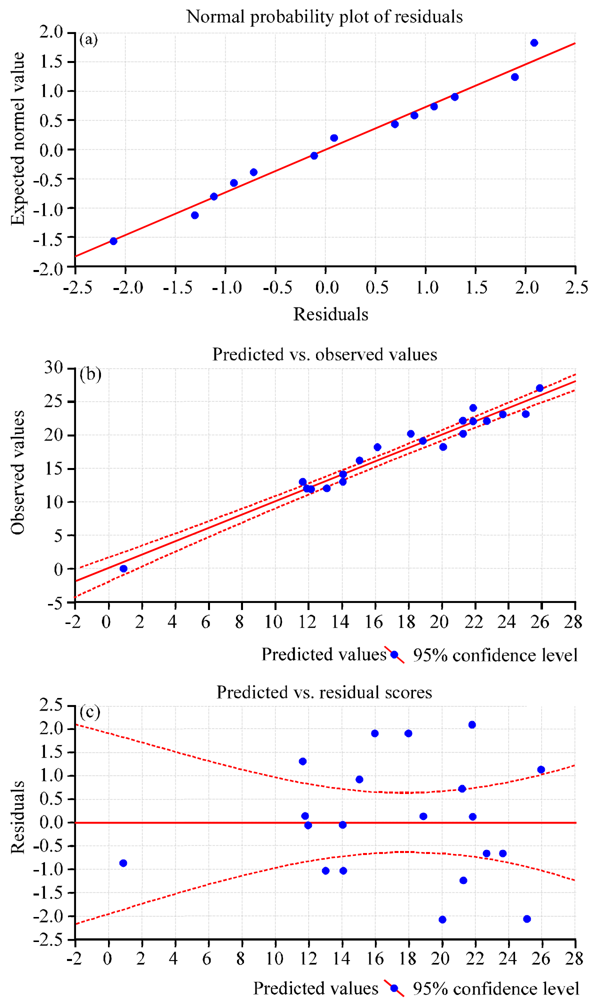

Fig. 4(a-c): (a) Normal probability plot of the residuals, (b) Correlation between the experimented and predicted values for antimicrobial metabolites activity of *Streptomyces anulatus* NEAE-94 determined by the first-order polynomial equation and (c) Plot of residuals against fitted values for antimicrobial metabolites production

normal probability plot of the residuals shows the points close to a diagonal line; therefore, the residuals appear to be approximately normally distributed. An excellent normal distribution confirmed the normality assumption and the independence of the residuals. This indicates that the model was well fitted with the experimental results. As the residuals from the fitted model are normally distributed, all the major assumptions of the model have been validated. Figure 4b which presents a plot of predicted vs. observed values of response, showed a satisfactory correlation between the experimental values and predicted values wherein, the points gathered around

Table 7: Box-Behnken experimental design, representing the response of antimicrobial metabolites activity as influenced by agitation speed (X_1), inoculum size (X_2) and inoculum age (X_3) along with the predicted antimicrobial metabolites activities and residuals

	Variables			Inhibition zone (mm)		
Trials	X_1	X_2	X_3	Experimental	Predicted	Residuals
1	-1	-1	0	17	14.25	2.75
2	1	-1	0	36	34.75	1.25
3	-1	1	0	26	27.25	-1.25
4	1	1	0	26	28.75	-2.75
5	-1	0	-1	31	32.00	-1.00
6	1	0	-1	37	36.50	0.50
7	-1	0	1	14	14.50	-0.50
8	1	0	1	33	32.00	1.00
9	0	-1	-1	20	21.75	-1.75
10	0	1	-1	7	09.25	-2.25
11	0	-1	1	26	23.75	2.25
12	0	1	1	16	14.25	1.75
13	0	0	0	21	21.00	0.00
14	0	0	0	21	21.00	0.00
15	0	0	0	21	21.00	0.00

the diagonal line indicates the good fit of the model. The residual plot in Fig. 4c shows equal scatter of the residual data above and below the x-axis, indicating that the variance was independent of antimicrobial metabolites production, thus supporting the adequacy of the model fit.

In a confirmatory experiment, to evaluate the accuracy of Plackett-Burman design, a medium which expected to be near optimum of the following composition (g L^{-1}): starch 20, KNO_3 2, K_2HPO_4 0.5, NaCl 0.1, $MgSO_4.7H_2O$ 0.1, $CaCO_3$ 3, $FeSO_4.7H_2O$ 0.01, pH 6.5, temperature 25°C, agitation speed of 200 rpm min^{-1}, medium volume 75 mL, inoculum size 4% (v/v), inoculum age 60 h and incubation period 5 days, gives 30 mm inhibition zone which is higher than result obtained from the basal medium before applying Plackett-Burman by 1.5 times (20 mm inhibition zone).

Optimization by response surface methodology: The Box-Behnken design was employed to study the interactions among the significant factors those had positive effect on the antimicrobial metabolites production and also determine their optimal levels. The other variables in the study were maintained at a constant level which gave maximal yield in the Plackett-Burman experiments. A total of 15 experiments with different combination of agitation speed (rpm min^{-1}), inoculum size (%) and inoculum age (h) were performed and the results of experiments are represented in Table 7. The results showed considerable variation in the antimicrobial metabolites activity. Treatment runs from 2 to 6, 8 and 11 showed a high antimicrobial metabolites activity. The maximum antimicrobial metabolites activity (37 mm) was achieved in run number 6 while the minimum antimicrobial metabolites activity (7 mm) was observed in run number 10.

Multiple regression analysis and ANOVA: Multiple regression analysis was used to analyze the data, the goodness of fit of the model was checked by the coefficient of determination (R^2) which was found to be 0.9628, indicating that the sample variation of 96.28% was attributed to the variables and only 3.72% of the total variance could not be explained by the model. A regression model having an R^2-value higher than 0.9 was considered as having a very high correlation (Chen *et al.*, 2009). Therefore, the present R^2-value reflected a very good fit between the observed and predicted responses and implied that the model is reliable for antimicrobial metabolites production in the present study. In Table 7 each of the observed values for antimicrobial agent is compared with the predicted values, from the model.

Analysis of variance (ANOVA) which is required to test the significance and adequacy of the model is presented in Table 8. The analysis of variance (ANOVA) of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test (14.395) and a very low probability value (0.0045). The significance of each coefficient was determined by t-values and p-values which are listed in Table 9. The p-values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables. It can be seen from the degree of significance that the linear coefficients of X_1 (agitation speed), X_3 (inoculum age) and quadratic effect of X_1 are significant, meaning that they can act as limiting factor and little variation in their value will alter the product production rate. Furthermore, the probability values of the coefficient suggest that among the three variables studied, X_1 (agitation speed) and X_2 (inoculum size) showed maximum interaction between the two variables (0.017), indicating that 98.3% of the model affected by these variables. On the other hand, among the different interactions, interaction between X_2 (inoculum size) and X_3 (inoculum age) and quadratic effect of X_3 did not show significant effect on antimicrobial metabolites production.

In order to evaluate the relationship between dependent and independent variables and to determine the maximum antimicrobial metabolites production corresponding to the optimum levels of agitation speed (X_1), inoculum size (X_2) and inoculum age (X_3), a second-order polynomial model (Eq. 5) was proposed to calculate the optimum levels of these variables. By applying the multiple regression analysis on experimental data, the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_2 and X_3) was obtained:

$$Y_{(\text{Antimicrobial metabolites production})} = 21 + 5.5\,X_1 + 1.75\,X_2 - 5.5\,X_3 - 4.75\,X_1X_2 + 3.25\,X_1X_3 + 0.75\,X_2X_3 + 8.375\,X_1^2 - 3.125\,X_2^2 - 0.625\,X_3^2 \quad (5)$$

where, the Y is the predicted response, X_1 the coded value of agitation speed, X_2 the coded value of inoculum size and X_3 the coded value of inoculum age.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves (Fig. 5a-c) when one of the variables is fixed at optimum value and the other two are allowed to vary. Figure 5a represents the interaction between

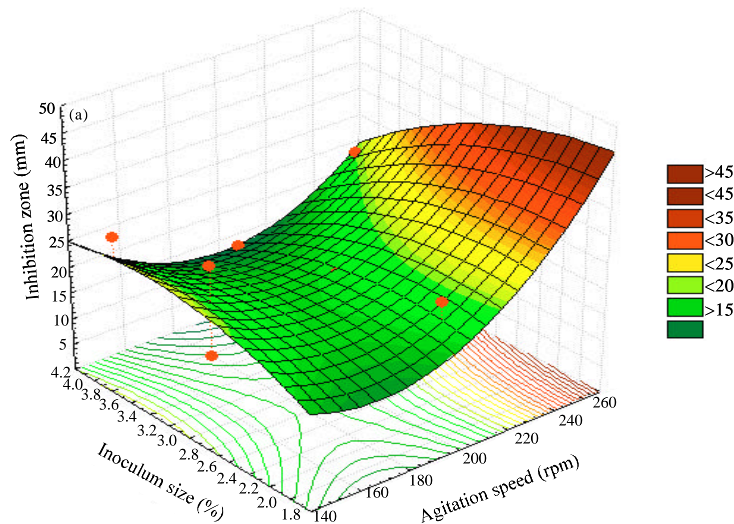

Fig. 5(a-c): Continue

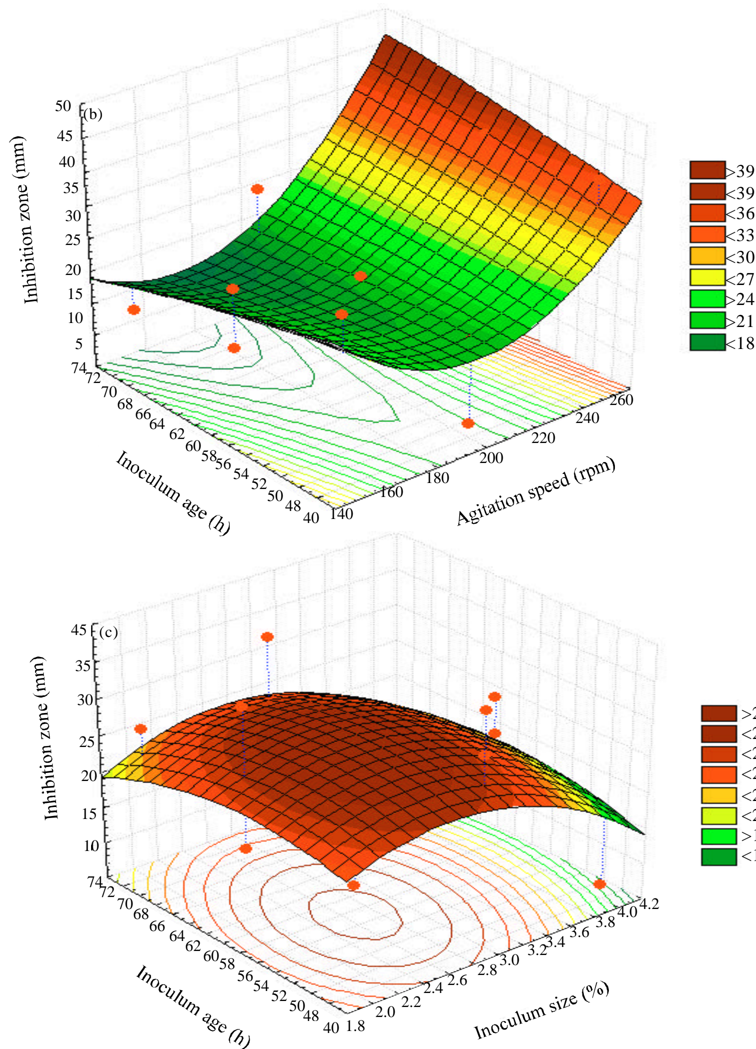

Fig. 5(a-c): Three-dimensional response surface plots showing the interactive effects of independent variables (a) Agitation speed (rpm), (b) Inoculum size (%, v/v) and (c) Inoculum age (h) on antimicrobial metabolites activity

Table 8: Analysis of variance (ANOVA) for optimization of antimicrobial metabolites activity using Box-Behnken design

Varaibles	df	SS	MS	F	Significance F
Regression	9	958.733	106.526	14.395	0.0045
Residual	5	37.000	7.400		
Total	14	995.733			

df: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares, F: Fishers's function, Significance F: Corresponding level of significance

agitation speed (X_1), inoculum size (X_2). It showed that low level of inoculum size and high level of agitation speed supported high antimicrobial metabolites activity. Figure 5b showed that the highest value of antimicrobial metabolites activity was obtained with high agitation speed and low or middle level of inoculum age. Further increase of inoculum age did not result in higher antimicrobial metabolites yields. In the interaction studies of inoculum size and inoculum age, the maximum

Table 9: Estimated regression coefficients, main effect, t-test and p-values for optimization of antimicrobial metabolites activity using Box-Behnken design (RSM)

Variables	Coefficients	Main effect	t-stat	p-value
Intercept	21.000	42.00	13.371	0.000
X_1	5.500	11.00	5.719	0.002
X_2	1.750	3.50	1.820	0.128
X_3	-5.500	-11.00	-5.719	0.002
X_1X_2	-4.750	-9.50	-3.492	0.017
X_1X_3	3.250	6.50	2.389	0.062
X_2X_3	0.750	1.50	0.551	0.605
X_1X_1	8.375	16.75	5.916	0.002
X_2X_2	-3.125	-6.25	-2.207	0.078
X_3X_3	-0.625	-1.25	-0.441	0.677

t: Student's test; p: Corresponding level of significance

antimicrobial metabolites production was attained at low to moderate levels of both the factors while further increase in the levels resulted in a gradual decrease in yield (Fig. 5c).

Verification of the model: Optimal concentrations of the factors, obtained from the optimization experiment were verified experimentally and compared with the predicted data. The measured antimicrobial metabolites activity was 37 mm, where the predicted value from the polynomial model was 37.6 mm. The verification revealed a high degree of accuracy of the model of more than 98.85%, indicating the model validation under the tested conditions. The optimal levels of the process variables for antimicrobial metabolites production by *Streptomyces anulatus* NEAE-94 were agitation speed (250 rpm min^{-1}), inoculum size (4%, v/v) and inoculum age (60 h).

CONCLUSION

This study proved that statistical experimental design showed significant results for optimizing the process parameters for maximum antimicrobial metabolites production under submerged fermentation using *Streptomyces anulatus* NEAE-94 and allowed rapid screening of a large number of variables. The antimicrobial metabolites production was found to be significantly influenced by agitation speed, inoculum size and inoculum age. A maximum extracellular antimicrobial metabolites production of 37 mm was achieved with the following optimized factors: Starch 20 g, KNO_3 2 g, K_2HPO_4 0.5 g, NaCl 0.1 g, $MgSO_4.7H_2O$ 0.1 g, $CaCO_3$ 3 g, $FeSO_4.7H_2O$ 0.01 g, pH 6.5, temperature 25°C, agitation speed 250 rpm min^{-1}, medium volume 75 mL, inoculum size 4% (v/v), inoculum age 60 h and fermentation period 5 days. Validation experiments were also carried out to verify the adequacy and the accuracy of the model and results showed that the predicted value agreed with the experimental values well and about 2-fold increase compared to the original medium was obtained.

REFERENCES

Abdel-Fatah, M.K., 1996. Studies on the antifungal activities of streptomycetes. II- Nutritional and environmental conditions affecting the antifungal activity of *Streptomyces prunicolor* (W-5). Egypt. J. Microbiol., 31: 437-455.

Aghaie-Khouzani, M., H. Forootanfar, M. Moshfegh, M.R. Khoshayand and M.A. Faramarzi, 2012. Decolorization of some synthetic dyes using optimized culture broth of laccase producing ascomycete *Paraconiothyrium variabile*. Biochem. Eng. J., 60: 9-15.

Akhnazarova, S. and V. Kafarov, 1982. Experiment Optimization in Chemistry and Chemical Engineering. Mir Publication, Moscow.

Arasu, M.V., V. Duraipandiyan, P. Agastian and S. Ignacimuthu, 2008. Antimicrobial activity of *Streptomyces* spp. ERI-26 recovered from Western Ghats of Tamil Nadu. J. Med. Mycol., 18: 147-153.

Baltz, R.H., 1998. Genetic manipulation of antibioticproducing *Streptomyces*. Trends Microbiol., 6: 76-83.

Beg, Q.K., V. Sahai and R. Gupta, 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in bioreactor. Process Biochem. 39: 203-209.

Box, G.E.P. and D.W. Behnken, 1960. Some new three level designs for the study of quantitative variables. Technometrics, 2: 455-475.

Box, G.E.P., W.G. Hunter and J.S. Hunter, 1978. Statistics for Experiments. Wiley, New York, USA., pp: 291-334.

Chen, X.C., J.X. Bai, J.M. Cao, Z.J. Li and J. Xiong *et al*., 2009. Medium optimization for the production of cyclic adenosine 3',5'-monophosphate by *Microbacterium* sp. no. 205 using response surface methodology. Bioresour. Technol., 100: 919-924.

Deepak, V., K. Kalishwaralal, S. Ramkumarpandian, S. Venkatesh Babu, S.R. Senthilkumar and G. Sangiliyandi, 2008. Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. Bioresour. Technol., 99: 8170-8174.

DermNet NZ., 2013. Staphylococcal skin infections. http://www.dermnetnz.org/bacterial/staphylococci.html

Dhanasekaran, D., S. Selvamani, A. Panneerselvam and N. Thajuddin, 2009. Isolation and characterization of actinomycetes in Vellar Estuary, Annagkoil, Tamil Nadu. Afr. J. Biotechnol., 8: 4159-4162.

El-Naggar, M.Y., M.A. Hassan, W.Y. Said and S.A. El-Assar, 2003. Effect of support materials on antibiotic MSW2000 production by immobilized *Streptomyces violatus*. J. Gen. Applied Microbiol., 49: 235-243.

Ettler, P., 1992. The determination of optimal inoculum quality for submerase fermentation process. Czech. Chem. Commun., 57: 303-308.

Gangadharan, D., S. Sivaramakrishnan, K.M. Nampoothiri, R.K. Sukumaran and A. Pandey, 2008. Response surface methodology for the optimization of α amylase production by *Bacillus amyloliquefaciens*. Bioresour. Technol., 99: 4597-4602.

Gao, H., M. Liu, J. Liu, H. Dai and X. Zhou *et al*., 2009. Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology. Biores. Technol., 100: 4012-4016.

Gill, P.K., A.D. Sharma, R.K. Harchand and P. Singh, 2003. Effect of media supplements and culture conditions on inulinase production by an actinomycete strain. Bioresour. Technol., 87: 359-362.

Goodfellow, M., S.T. Williams and M. Mordarski, 1988. Actinomycetes in Biotechnology. Academic Press, London, New York, pp: 78-82.

Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser and C.J. Bruton *et al*., 1985. Genetic Manipulation of Streptomyces: A Laboratory Manual. The John Innes Foundation, Norwich, United Kingdom, ISBN: 0-7084-0336-0, pp: 79-80.

Hotta, K. and Y. Okami, 1996. Diversity in aminoglycoside antibiotic resistance of actinomycetes and its exploitation in the search for novel antibiotics. J. Ind. Microbiol., 17: 352-358.

Jiang, Y., B. Ji, R.N.S. Wong and M.H. Wong, 2008. Statistical study on the effects of environmental factors on the growth and microcystins production of bloom-forming cyanobacterium-*Microcystis aeruginosa*. Harmful Algae, 7: 127-136.

Kaushik, R., S. Saran, J. Isar and R.K. Saxena, 2006. Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. J. Mol. Catal. B Enzym., 40: 121-126.

Khanna, S. and A.K. Srivastava, 2005. Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process Biochem., 40: 2173-2182.

Kieser, T., M.J. Bibb, M.J. Buttner, K.F. Chater and D.A. Hopwood, 2000. Practical Streptomyces Genetics. John Innes Foundation, Norwich, UK.

Krishnan, S., S.G. Prapulla, D. Rajalakshmi, M.C. Misra and N.G. Karanth, 1998. Screening and selection of media components for lactic acid production using Plackett-Burman design. Bioprocess Eng., 19: 61-65.

Kumar, C.G. and H. Takagi, 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv., 17: 561-594.

Li, Y., H. Jiang, Y. Xu and X. Zhang, 2008. Optimization of nutrient components for enhanced phenazine-1-carboxylic acid production by gacA-inactivated *Pseudomonas* sp. M18G using response surface method. Applied Microbiol. Biotechnol., 77: 1207-1217.

Liu, G.Q. and X.L. Wang, 2007. Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei*. Applied Microbiol. Biotechnol., 74: 78-83.

Lopes, J.A., J.C. Menezes, J.A. Westerhuis and A.K. Smilde, 2002. Multiblock PLS analysis of an industrial pharmaceutical process. Biotechnol. Bioeng., 80: 419-427.

Mahalaxmi, Y., T. Sathish, C. Subba Rao and R.S. Prakasham, 2010. Corn husk as a novel substrate for the production of rifamycin B by isolated *Amycolatopsis* sp. RSP 3 under SSF. Proc. Biochem., 45: 47-53.

Montgomery, D.C., 1991. Design and Analysis of Experiments. John Wiley and Sons Inc., New York.

Mudgetti, R.E., L.D. Arnolds and A.S. Nadine, 1986. Manual of Industrial Biotechnology. American Society for Microbiology, Washington, DC., Pages: 66.

Muruganandham, M., B. Eswarapriya, B. Gopalsamy, S. Kalidas and B. Kameswari *et al*., 2010. Calciferol influence on pathogenicity against *Staphylococuus aureus*. J. Global Pharma. Technol., 2: 58-65.

Neves, A.A., L.M. Vieira and J.C. Menezes, 2000. Effects of preculture variability on clavulanic acid fermentation. Biotechnol. Bioeng., 72: 628-633.

Olano, C., F. Lombo, C. Mendez and J.A. Salas, 2008. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metab. Eng., 10: 281-292.

Plackett, R.L. and J.P. Burman, 1946. The design of optimum multifactorial experiments. Biometrika, 33: 305-325.

Rahman, R.N., P.L. Geok, M. Basri and A.B. Salleh, 2005. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. Bioresour. Technol., 96: 429-436.

Raja, A. and P. Prabakarana, 2011. Actinomycetes and drug-An overview. Am. J. Drug Discovery Dev., 1: 75-84.

Ramachandran, S., A.K. Patel, K.M. Nampoothiri, F. Francis, V. Nagy, G. Szakacs and A. Pandey, 2004. Coconut oil cake: A potential raw material for the production of α-amylase. Bioresour. Technol., 93: 169-174.

Ren, J., W.T. Lin, Y.J. Shen, J.F. Wang, X.C. Luo and M.Q. Xie, 2008. Optimization of fermentation media for nitrite oxidizing bacteria using sequential statistical design. Bioresour. Technol., 99: 7923-7927.

Sahin, N. and A. Ugur, 2003. Investigation of the antimicrobial activity of some *Streptomyces* isolates. Turk. J. Biol., 27: 79-84.

Sayyad, S.A., B.P. Panda, S. Javed and M. Ali, 2007. Optimization of nutrient parameters for lovastatin production by *Monascus purpureus* MTCC 369 under submerged fermentation using response surface methodology. Applied Microbiol. Biotechnol., 73: 1054-1058.

Stowe, R.A. and R.P. Mayer, 1966. Efficient screening of process variables. Ind. Eng. Chem., 58: 36-40.

Vecht-Lifshitz, S.E., S. Magdassi and S. Braun, 1989. Effects of surface active agents on pellet formation in submerged fermentations of *Streptomyces tendae*. J. Dispersion Sci. Technol., 10: 265-275.

Waksman, S.A., 1959. Strain specificity and production of antibiotic substances. X. characterization and classification of species within the *Streptomyces griseus* group. Proc. Nat. Acad. Sci. USA., 45: 1043-1043.

Wang, Y.H., J.T. Feng, Q. Zhang and X. Zhang, 2008. Optimization of fermentation condition for antibiotic production by *Xenorhabdus nematophila* with response surface methodology. J. Appl. Microbiol., 104: 735-744.

Wang, Y.X. and Z.X. Lu, 2004. Statistical optimization of media for extracellular polysaccharide by *Pholiota squarrosa* (Pers. ex Fr.) Quel. AS 5.245 under submerged cultivation. Biochem. Eng. J., 20: 39-47.

Watve, M., R. Tickoo, M.M. Jog and B.W. Bhole, 2001. How many antibiotics are produced by the genus *Streptomyces*? Arch. Microbiol., 176: 386-390.

Woolford, M.K., 1972. The Semi large-scale production, extraction, purification and properties of an antibiotic produced by *Bacillus licheniformis* strain 2725. J. Applied Microb., 35: 227-231.

Yu, X., S.G. Hallet, J. Sheppard and A.K. Watson, 1997. Application of the Plackett-Burman experimental design to evaluate nutritional requirements for the production of *Colletotrichum coccodes* spores. Applied Microbiol. Biotechnol., 47: 301-305.