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Production of Antimicrobial Agent Inhibitory to some Human Pathogenic Multidrug-Resistant Bacteria and *Candida albicans* by *Streptomyces* sp. NEAE-1

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Abstract: The production of antimicrobial agent by *Streptomyces* sp. NEAE-1 in shake flask culture was optimized using response surface methodology. Initial screening of production parameters was performed using a Plackett-Burman design and the variables with statistically significant effects on antimicrobial agent production were identified. Out of the fifteen factors screened, KNO_3, NaCl and inoculum size were selected due to significant positive effect on the production of antimicrobial agent. The optimal levels of these variables and the effect of their mutual interactions on antimicrobial agent production were determined using Box-Behnken design. The maximum antimicrobial agent activity was achieved at the KNO_3 (3 g L^{-1}), NaCl (0.3 g L^{-1}), inoculum size (4%, v/v). The statistical optimization by response surface methodology resulted in more than one and half-fold increase in the production of antimicrobial agent by *Streptomyces* sp. NEAE-1. The maximal antimicrobial agent activity is 27 mm inhibition zones which is achieved at the following fermentation conditions: g L^{-1} (starch 10 g, KNO_3 3 g, K_2HPO_4 0.5 g, NaCl 0.3 g, $MgSO_4.7H_2O$ 0.5 g, $CaCO_3$ 1 g, $FeSO_4.7H_2O$ 0.01 g), pH 6.5, temperature 25°C, agitation speed (200 rpm min^{-1}), medium volume 50 mL, inoculum size 4% (v/v), inoculum age 60 h and fermentation period 7 days. The value of the coefficient of determination (R^2) for the production of antimicrobial agent was 0.955 which indicates a good agreement between experimental and predicted values.

Key words: *Streptomyces* sp. NEAE-1, antimicrobial metabolites, Plackett-Burman design, Box-Behnken design, multidrug-resistant *Staphylococcus aureus*

INTRODUCTION

Misuse and overuse of antibiotics can create the conditions for the development of antibiotic resistant bacteria. Antimicrobial resistance among gram-positive organisms has been increasing steadily during the past several decades (Novak *et al*., 1999). The increase in the frequency of multi-resistant pathogenic bacteria is created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity which resist the inactivation processes exploited by microbial enzymes (Motta *et al*., 2004).

Microorganisms are virtually unlimited sources of novel compounds with many medicinal and agricultural applications. Among all the known microbes, the genus *Streptomyces* is one of prominent soil inhabitant, comprising up to 90% of actinomycetes isolated from soil samples. *Streptomyces* is the largest and the most important genus in the order actinomycetales. Members of the genus *Streptomyces* are prolific producers of bioactive secondary metabolites that have important applications both in medicine and agriculture (Atta and Ahmad, 2009; Atta *et al*., 2010; Demain and Sanchez, 2009). Many drugs have been developed from *Streptomyces* spp. belonging to different classes of antibiotics such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines (Murray, 2011; Baltz, 2007; Watve *et al*., 2001).

Since antibiotics are secondary metabolites synthesized by pathways which are often connected and influenced by primary metabolism, thus, frequently an intermediate metabolite from primary metabolism serves as precursor for the biosynthesis of the antibiotic. Therefore, the medium constitution together with the metabolic capacity of the producing organism greatly influences the

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biosynthesis of antibiotics (El-Refai *et al*., 2011). The ability of streptomycete cultures to form antibiotics is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation (Waksman, 1961). Thus, the optimization of fermentation conditions, particularly physical and chemical parameters, is important in the development of fermentation processes and thus increases the yield of antibiotic without increasing the cost of production.

Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one parameter at a time while keeping the others at a fixed level (Liu and Tzeng, 1998). The optimization studies do not consider the interaction effects among the variables as any process is influenced by several variables (Silva and Roberto, 2001). In contrast, experimental design offers a number of important advantages as the researchers could easily determine effects of factors with considerably less experimental effort, find real optimum value and facilitate system modeling (Bandaru *et al*., 2006). The statistical designs such as the Plackett-Burman design and response surface method are effective methods for optimization of the operational parameters (Wang and Liu, 2008).

In the present study, a Placket-Burman experimental design was used to screen the significant process variables that influencing the antimicrobial agent production by *Streptomyces* sp. NEAE-1. Then the Box-Behnken statistical design was further applied to determine the optimum level of each significant variables.

MATERIALS AND METHODS

Microorganisms and cultural conditions: *Streptomyces* sp. NEAE-1 was kindly provided by Dr. Noura El-Ahmady El-Naggar (Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technologial Applications, Alexandria, Egypt). *Streptomyces* sp. NEAE-1, an antagonistic actinomycete newly isolated from Egyptian soil, exhibited a broad antimicrobial spectrum against several microorganisms. 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 1 L. 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), *Proteus vulgaris* (this strain is resistant to Piperacillin, Ampo-sulbactam, Cefuroxime and chloramphenicol) and *Klebsiella pneumonia* A9898 (resistant to Trimethoprim-sulfamethoxazole, Augmentin, Gentamicin, Ceftriaxone, Amikacin and Cefotaxime); a group of bacteria belonging to the culture collection of NRRL: Gram-positive (*Staphylococcus aureus* NRRL B-313, *Bacillus subtilis* NRRL B-543) and Gram-negative (*Escherichia coli* NRRL B-210, *Pseudomonas aeruginosa* NRRL B-23). 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: The 250 mL 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 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 *Candida 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 Plackett-Burman statistical experimental design is a two factorial design which identifies the critical physico-chemical parameters required for elevated antimicrobial agent production and is 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 and Burman (1946) is n+1, where n is the number of variables. 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:

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

where, Y is the response or dependent variable (antimicrobial agent activity); it will always be the variable we aim to predict, β_0 is the model intercept and β_i is the linear coefficient and X_i is the level of the independent variable; it is the variable that will help us explain antimicrobial agent activity.

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 agent activities were treated as responses.

Response surface methodology (RSM): The levels and the interaction effects between various significant variables which exerted a positive effect on the antimicrobial agent 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:

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

		Levels	
Independent variables	Code	-1	+1
Starch (g L^{-1})	X_1	10	20
KNO_3 (g L^{-1})	X_2	1	2
K_2HPO_4 (g L^{-1})	X_3	0.5	1
Yeast extract (g L^{-1})	X_4	0	0.1
NaCl (g L^{-1})	X_5	0.1	0.5
$MgSO_4.7H_2O$ (g L^{-1})	X_6	0.1	0.5
$CaCO_3$ (g L^{-1})	X_7	1	3
$FeSO_4$ (g L^{-1})	X_8	0.01	0.02
pH	X_9	6.5	7.5
Temperature (°C)	X_{10}	25	30
Agitation speed (rpm min^{-1})	X_{11}	150	200
Medium volume (mL 250 mL^{-1} flask)	X_{12}	50	75
Inoculum size (%, v/v)	X_{13}	2	4
Inoculum age (h)	X_{14}	48	60
Fermentation time (d)	X_{15}	5	7

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

Variable	Variable code	-1	0	1
KNO_3 (g L^{-1})	X_1	1.0	2.0	3 .0
NaCl (g L^{-1})	X_2	0.3	0.5	0.7
Inoculum size (%, v/v)	X_3	2.0	4.0	6.0

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

where, 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 X_i 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:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (3)$$

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 the antimicrobial agent was tested against Gram-negative, Gram-positive bacteria and *Candida albicans* (Table 3). It has a strong activity against multidrug resistant *Staphylococcus aureus* A9897 and *Staphylococcus aureus* NRRL B-313. The clear zone diameter obtained with multidrug resistant *Proteus vulgaris*, *Staphylococcus aureus* NRRL B-313, multidrug resistant *Staphylococcus aureus* A9897 and *Bacillus subtilis* NRRL B-543 were 20, 20, 16, 15 mm, respectively. There is no activity against multidrug resistant *Klebsiella pneumonia* A9898. While the clear zone diameter 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, 22, 17, 19 mm, respectively.

Screening of parameters using Plackett-Burman design: The design matrix selected for the screening of significant variables for antimicrobial agent production and the corresponding responses are shown in Table 4. The experiment was conducted in 20 runs to study the effect of the selected variables on the production of antimicrobial agent. The mycelial growth has been shown as small, red spherical pellets (Fig. 1) during the antimicrobial metabolites production in shake flasks. In submerged cultures, *Streptomyces* tends to form fluffy

Table 3: Antimicrobial activity of the antimicrobial agent produced by *Streptomyces* sp. NEAE-1

Microorganisms	Specification	Inhibition zone diameter (mm)
Gram positive bacteria		
Proteus vulgaris	Resistance to piperacillin, ampi-sulbactam, cefuroxime and chloramphenicol	20
Staphylococcus aureus	NRRL B-313	20
Staphylococcus aureus	A9897, resistant to vancomycin, augmentin, gentamicin, trimethoprim-sulfamethoxazole, Oxacillin, amikacin and tobramycin	16
Bacillus subtilis	NRRL B-543	15
Gram negative bacteria		
Escherichia coli	NRRL B-210	18
Pseudomonas aeruginosa	NRRL B-23	22
Pseudomonas aeruginosa	T9934, resistant to ceftriaxone, gentamicin, cefotaxime, trimethoprim-sulfamethoxazole and augmentin	17
Klebsiella pneumonia	A9898, resistant to trimethoprim-sulfamethoxazole, augmentin, gentamicin, ceftriaxone, amikacin and cefotaxime	Negative
Yeast		
Candida albicans	NRRL Y-477	19

Table 4: Twenty-trial Plackett-Burman experimental design for evaluation of fifteen independent variables with coded values along with the observed antimicrobial agent 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	14
2	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	15
3	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	13
4	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	16
5	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	19
6	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	20
7	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	25
8	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	20
9	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	19
10	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	14
11	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	13
12	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	18
13	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	23
14	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	19
15	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	14
16	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	20
17	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	21
18	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	0
19	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	17
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	16

-1: Low level, +1: High level

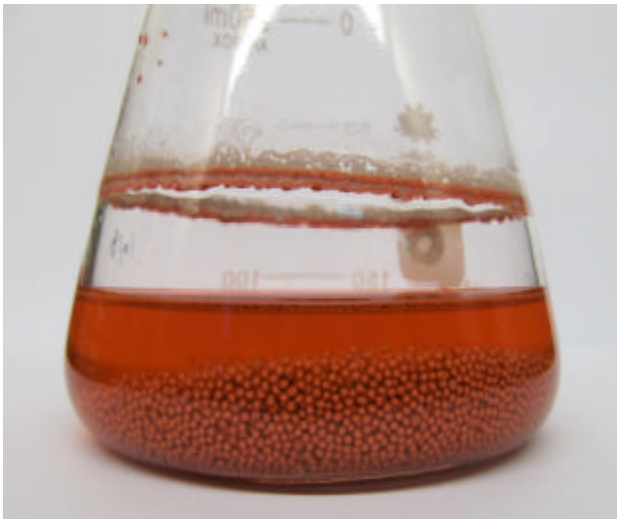

Fig. 1: *Streptomyces* sp. NEAE-1 growth in red spherical pellets form during the antimicrobial agent production in shake flasks after inoculum and incubation on a rotary shaker (200 rpm) at 30°C

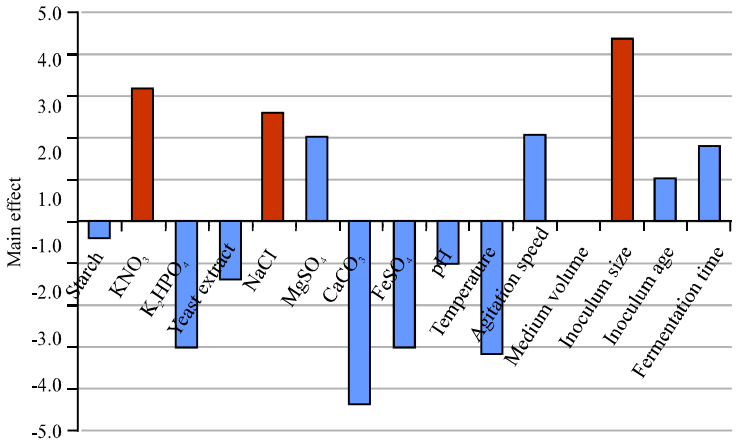

Fig. 2: Main effects of the fermentation medium constituents on antimicrobial agent production according to the Packett-Burman experimental results (the red color represent the most significant variables with positive effect)

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 responses were performed which is represented in Table 5. With respect to the main effect of each variable (Fig. 2), we can see that seven variables from the fifteen named KNO_3, NaCl, $MgSO_4$, agitation speed, inoculum size, inoculum age and fermentation time positively affect antimicrobial agent production, where the other seven variables named starch, K_2HPO_4, yeast extract, $CaCO_3$, $FeSO_4$, pH and temperature negatively affect antimicrobial agent production while medium volume is found to has no significant effect on the antimicrobial agent production. The variables with positive effect were fixed at high level. Starch, K_2HPO_4, $CaCO_3$, $FeSO_4$, pH, temperature and medium volume were maintained at their low (-1) level while yeast extract will be excluded in the subsequent experiment. The Pareto chart illustrates the order of significance of the variables affecting antimicrobial agent production (Fig. 3). Among the 15 variables, inoculum size showed the high positive effect by 13.174%, also $CaCO_3$ showed the high negative significance by 13.174%.

The relationship between a set of independent variables and the response (Y) is determined by a mathematical model called multiple-regression model. How

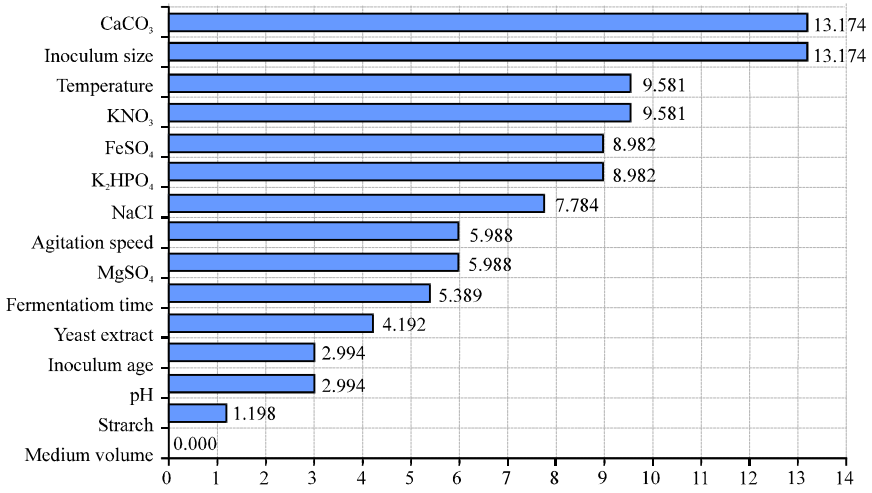

Fig. 3: Pareto chart illustrates the order of significance of the variables affecting the antimicrobial agent production by *Streptomyces* sp. NEAE-1

Table 5: Statistical analysis of Plackett-Burman design showing coefficient values, t-test, p-values and confidence level (%) for each variable affecting antimicrobial agent production

Variables	Coefficients	Main effect	t-Stat	p-value	Confidence level (%)
Intercept	16.8	33.6	42.332	0.000	99.9990
Starch (g L^{-1})	-0.2	-0.4	-0.504	0.641	35.9210
KNO_3 (g L^{-1})	1.6	3.2	4.032	0.016	98.4290
K_2HPO_4 (g L^{-1})	-1.5	-3.0	-3.780	0.019	98.0560
Yeast extract (g L^{-1})	-0.7	-1.4	-1.764	0.153	84.7470
NaCl (g L^{-1})	1.3	2.6	3.276	0.031	96.9380
$MgSO_4.7H_2O$ (g L^{-1})	1.0	2.0	2.520	0.065	93.4630
$CaCO_3$ (g L^{-1})	-2.2	-4.4	-5.543	0.005	99.4820
$FeSO_4$ (g L^{-1})	-1.5	-3.0	-3.780	0.019	98.0560
pH	-0.5	-1.0	-1.260	0.276	72.3790
Temperature (°C)	-1.6	-3.2	-4.032	0.016	98.4290
Agitation speed (rpm min^{-1})	1.0	2.0	2.520	0.065	93.4630
Medium volume (mL 250 mL^{-1} flask)	0.0	0.0	0.000	1.000	0.0000
Inoculum size (%, v/v)	2.2	4.4	5.543	0.005	99.4820
Inoculum age (h)	0.5	1.0	1.260	0.276	72.3790
Fermentation time (d)	0.9	1.8	2.268	0.086	91.4060

Multiple R: 0.9876, R square: 0.9753, Adjusted R square: 0.8825. t: Student's test; p: Corresponding level of significance

well the estimated model fits the data can be measured by the value of the coefficient of determination (R^2). The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental factors. The R^2 value is always between 0 and 1. When R^2 is closer to the 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 = 0.9753) indicates that 97.53% of the variability in the response was attributed to the given independent variables and only 2.47% of the total variations are not explained by the independent variables. In addition, the value of the adjusted determination coefficient (Adj. R^2 = 0.8825) is also very high which indicates a high significance of the model (Akhnazarova and Kafarov, 1982). A higher value of the correlation coefficient (R = 0.9876) signifies an excellent correlation between the independent variables (Box *et al*., 1978), this indicated a good correlation between the experimental and predicted values. Thus, the analysis of the response trend using the model was considered to be reasonable. The significance of each coefficient was determined by student's t-test and p-values which are listed in Table 5. 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).

The lower probability values indicate the more significant factors on the production of antimicrobial agent. Inoculum size and $CaCO_3$ with a probability value of 0.005 were determined to be the most significant

factors, followed by temperature and KNO_3 (0.016), K_2HPO_4 and $FeSO_4$ (0.019) and NaCl (0.031). Screened significant variables, KNO_3, NaCl and inoculum size, exerted positive effects whereas the other variables, $CaCO_3$, temperature, K_2HPO_4 and $FeSO_4$ exerted a negative effect on antimicrobial agent production. On the basis of the calculated t-values (Table 5), KNO_3 (X_2), NaCl (X_5) and inoculum size (X_{13}) were chosen for further optimization using Box-Behnken statistical design, since these factors had the most positive effects on the antimicrobial agent production.

More generally, several studies have shown that nitrogen assimilation is crucial for regulation of antibiotic production but the mechanisms involved have not yet been unraveled. In addition, there is experimental evidence for repression of antibiotic production exerted by some nitrogen sources and especially ammonium (Martin and Demain, 1980; Osman *et al*., 2011) showed that antimicrobial productivity by *Streptomyces plicatus* was greatly affected by the used nitrogen source KNO_3, $Ca(NO_3)_2$ and ammonium sulphate as a sole nitrogen sources in comparison to the other inorganic compounds and the highest productivity was in the case of KNO_3. El-Naggar *et al*. (2006) used starch nitrate medium containing 2 g L^{-1} potassium nitrate for the production of meroparamycin antibiotic by *Streptomyces* MAR01.

Perlman and Langlykke (1949) have shown that NaCl helps in the release of bound antibiotic from the mycelium, also salt concentration has a profound effect on the production of antibiotic from microorganism due to its effect on the osmotic pressure of the growth medium (Pelczar *et al*., 1993). El-Refai *et al*. (2011) was found that the antifungal activity of *Nocardioides luteus* was maximal, with an inhibition zone diameter of 37.7 mm, on using sodium chloride at concentration 1%. The antifungal activity at a greater NaCl concentration decreased to reach 30 mm at 4% concentration.

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 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 (Rahman *et al*., 2005; Woolford, 1972) found that 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. Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis (Mahalaxmi *et al*., 2010; Mukhtar *et al*., 2012) was found that 72 h old inoculum at a size of 4% (v/v) gave best antibiotic production. On the other hand, increase in anticandidal activity was not observed on using more than 2 mL 50 mL^{-1} production medium (El-Refai *et al*., 2011).

The model F-value of 10.5101 (Table 6) implies that the model is significant. The values of significance $F<0.05$ (0.0175) indicate model terms are significant. By neglecting the terms that were insignificant ($p>0.05$), the first order polynomial equation was derived representing antimicrobial agent production as a function of the independent variables:

$$Y_{(\text{Antimicrobial agent production})} = 16.8+1.6\ (X_2)-1.5\ (X_3)+ 1.3\ (X_5)-2.2\ (X_7)-1.5\ (X_8)-1.6\ (X_{10})+2.2\ (X_{13}) \quad (4)$$

where, Y is the response (antimicrobial agent production) and X_2, X_3, X_5, X_7, X_8, X_{10} and X_{13} are KNO_3, K_2HPO_4, NaCl, $CaCO_3$, $FeSO_4$, temperature and inoculum size; respectively. It can be seen from Eq. 4 that KNO_3, NaCl and inoculum size exerted positive effect on antimicrobial agent production by *Streptomyces* sp. NEAE-1.

Checking the adequacy of the model needs all of the information on lack of fit which is contained in the residuals. The normal probability plot of the residuals is an important diagnostic tool to detect and explain the systematic departures from the normality (Montgomery, 1991). Figure 4a shows a plot of normal probability of the experimental results. The normal probability plot is a graphical technique for assessing whether or not a data set is approximately normally distributed. The residual was plotted against a theoretical normal distribution of the model in such a way that the points should form an approximate straight line for antimicrobial agent production. Departures from this straight line indicate departures from normality. A linear pattern demonstrated that the errors are normally distributed and are independent of each other. The normal

Table 6: Analysis of variance (ANOVA) for optimization of antimicrobial agent production using Plackett-Burman design

Variables	df	SS	MS	F-test	Significance F (p-value)
Regression	15	496.6	33.1067	10.5101	0.0175
Residual	4	12.6	3.1500		
Total	19	509.2			

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

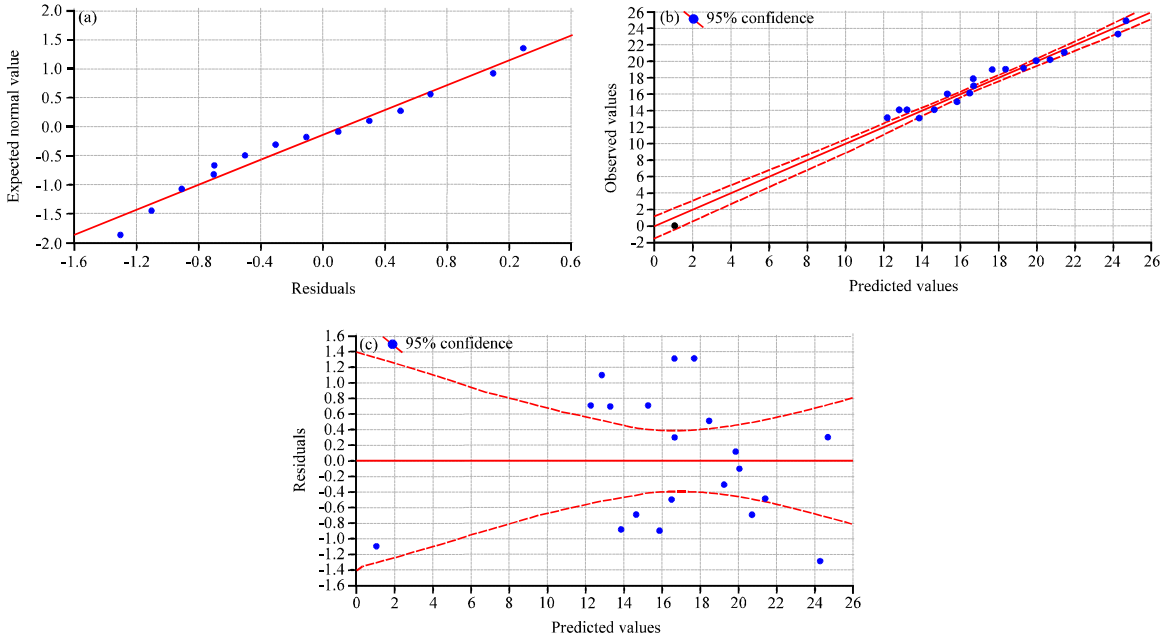

Fig. 4(a-c): (a) Normal probability plot of the residuals, (b) Correlation between the experimented and predicted values for antimicrobial agent activity of *Streptomyces* sp. NEAE-1 determined by the first-order polynomial equation and (c) Plot of residuals against predicted values for antimicrobial metabolites production

probability plot of the residuals shows the points close to a diagonal line; therefore, the residuals appear to be approximately normally distributed. This indicates that the model was well fitted with the experimental results. Figure 4b presents a plot of predicted vs. observed values of the response, showed a satisfactory correlation between the experimented values and predicted values wherein, the points gathered around 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, a medium which expected to be near optimum of the following composition (g L^{-1}): starch 10, KNO_3 2, K_2HPO_4 0.5, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 1, $FeSO_4.7H_2O$ 0.01, pH 6.5, temperature 25°C, agitation speed of 200 rpm min^{-1}, medium volume 50 mL, inoculum size 4% (v/v), inoculum age 60 h and incubation period 7 days, gives (25 mm) which is higher than result obtained from the basal medium before applying Plackett-Burman by more than one and one-half times (16 mm).

Optimization by response surface methodology: Based on the results of the Plackett-Burman experiments, the optimal levels and the interactions among the selected significant variables that influenced the antimicrobial agent production were analyzed and optimized by Box-Behnken methodology (Box and Behnken, 1960). In this study, A total of 15 experiments with different combination of KNO_3 (%), NaCl (%) and inoculum size (%, v/v) were performed and the results of experiments for studying the effects of three independent variables on antimicrobial agent activity are presented along with the observed, predicted response and residuals (Table 7).

Table 7: Box-Behnken experimental design, representing the response of antimicrobial agent activity as influenced by KNO_3 (X_1), NaCl (X_2) and inoculum size (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	26	26.0	0.0
2	1	-1	0	27	26.5	0.5
3	-1	1	0	26	26.5	-0.5
4	1	1	0	21	21.0	0.0
5	-1	0	-1	25	25.5	-0.5
6	1	0	-1	22	23.0	-1.0
7	-1	0	1	24	23.0	1.0
8	1	0	1	21	20.5	0.5
9	0	-1	-1	22	21.5	0.5
10	0	1	-1	21	22.0	-1.0
11	0	-1	1	23	22.0	1.0
12	0	1	1	16	16.5	-0.5
13	0	0	0	20	20.0	0.0
14	0	0	0	20	20.0	0.0
15	0	0	0	20	20.0	0.0

Treatment runs 1-3 and 5 showed a high antimicrobial agent activity (equal or more than 25 mm). The maximum antimicrobial agent activity (27 mm) was achieved in the run number 2 while the minimum antimicrobial agent activity (16 mm) was observed in the run number 12.

ANOVA and model fitting: The determination coefficient (R^2) of the model was 0.9545 indicating that 95.45% of variability in the response could be explained by the model and only 4.55% of the total variance could not be explained by the model. The highest R^2 value showed the good agreement between the experimental results and the theoretical values predicted by the model (Weisberg, 1985) and it showed that the model was suitable to represent the real relationship among the selected factors. 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 agent 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.

The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given 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 (11.659) and a very low probability value (0.0073).

All values of model coefficients were calculated by multiple regression analysis. The significance of each coefficient was determined by Student's t-test and p-values as listed in Table 9. The p-values were used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables (Pansuriya and Singhal, 2010). Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($p<0.05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient) (Moh *et al*., 2012).

It can be seen from the degree of significance that the linear coefficients of X_1 (KNO_3), X_2 (NaCl) and X_3 (inoculum size), quadratic effect of X_1 and X_2 are significant. These values suggest that the concentration of KNO_3 and NaCl have a direct relationship on the production of the antimicrobial agent. Positive

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

Variables	df	SS	MS	F-test	Significance F (p-value)
Regression	9	115.4333	12.8259	11.6599	0.0073
Residual	5	5.5000	1.1000		
Total	14	120.9333			

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

Table 9: Estimated regression coefficients for optimization of antimicrobial agent activity using Box-Behnken design (RSM)

Variables	Coefficients	Main effect	t-Stat	p-value
Intercept	20.00	40.0	33.029	0.0000
X_1	-1.25	-2.5	-3.371	0.0200
X_2	-1.25	-2.5	-3.371	0.0200
X_3	-1.25	-2.5	-3.371	0.0200
X_1X_2	-1.50	-3.0	-2.860	0.0350
X_1X_3	0.00	0.0	0.000	1.0000
X_2X_3	-1.50	-3.0	-2.860	0.0350
X_1X_1	3.75	7.5	6.870	0.0010
X_2X_2	1.25	2.5	2.290	0.0710
X_3X_3	-0.75	-1.5	-1.374	0.2280

Multiple R: 0.9770, R square: 0.9545, Adjusted R square: 0.8727. t:student's test; p:corresponding level of significance

coefficients for quadratic effect of KNO_3 (X_1) and NaCl (X_2) indicated a linear effect in the increase in antimicrobial agent production. Furthermore, the probability values of the coefficient suggest that among the three variables studied, X_1, X_2 and X_2, X_3 shows maximum interaction between the two variables (p-value 0.035), indicating that 96.5% of the model affected by these variables. On the other hand, among the different interactions, interaction between X_1 and X_3 were not significant (p-value >0.05), indicating that there is no significant correlation between each two variables and that they did not help much in increasing the production of antimicrobial agent

In order to evaluate the relationship between dependent and independent variables and to determine the maximum antimicrobial agent production corresponding to the optimum levels of KNO_3, NaCl and inoculum size, 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:

$$Y_{(\text{Antimicrobial agent production})} = 20-1.25\,X_1-1.25\,X_2-1.25\,X_3-1.5\,X_1X_2+0\,X_1X_3-1.5\,X_2X_3+3.75\,X_1^2+1.25\,X_2^2-0.75\,X_3^2 \quad (5)$$

where, the Y is the predicted response, X_1 the coded value of KNO_3, X_2 the coded value of NaCl and X_3 the coded value of inoculum size.

The three dimensional response surface curves were plotted by statistically significant model to understand the interaction of the variables and the optimal levels of each variable required for the optimal antimicrobial agent production (shown in Fig. 5a-c) when one of the variables is fixed at optimum value and the other two are allowed to

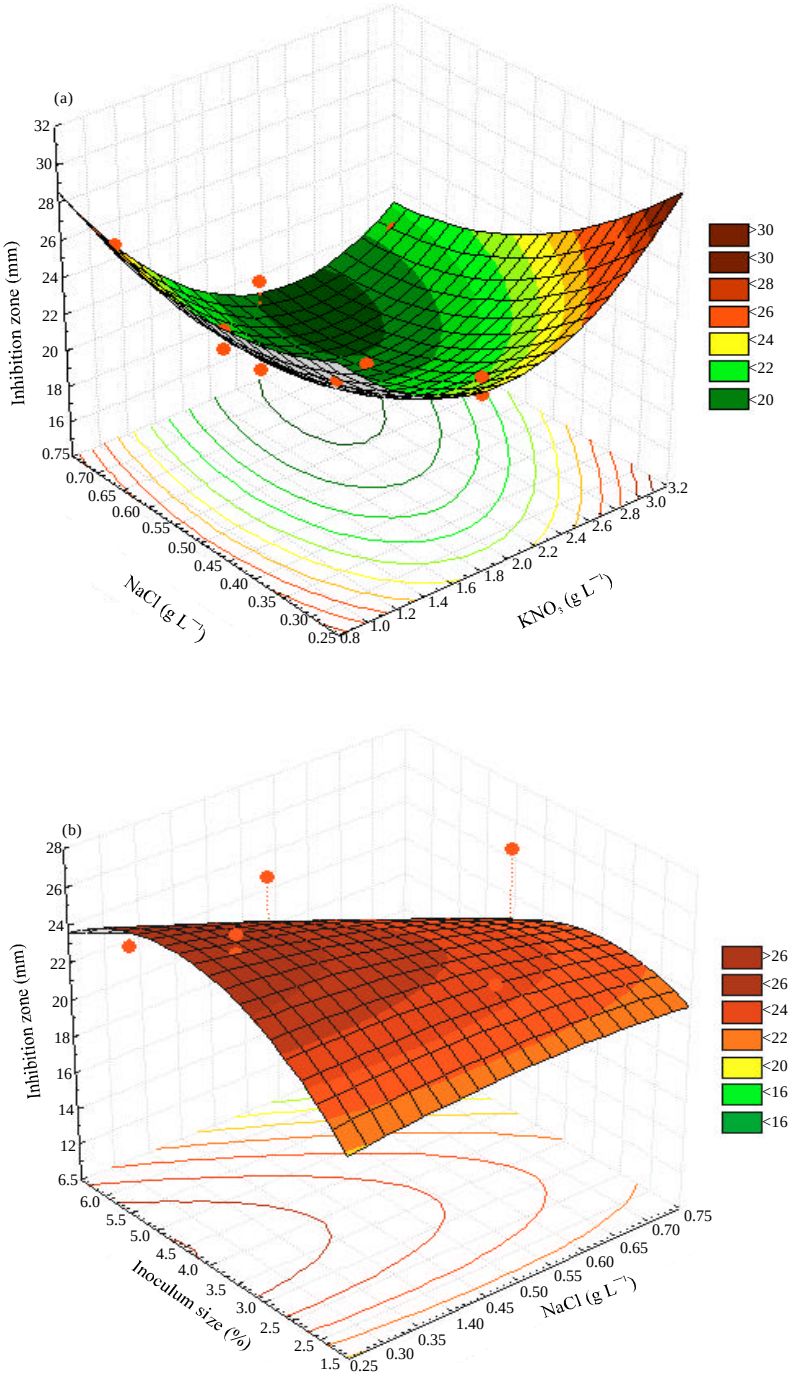

Fig. 5(a-c): Continue

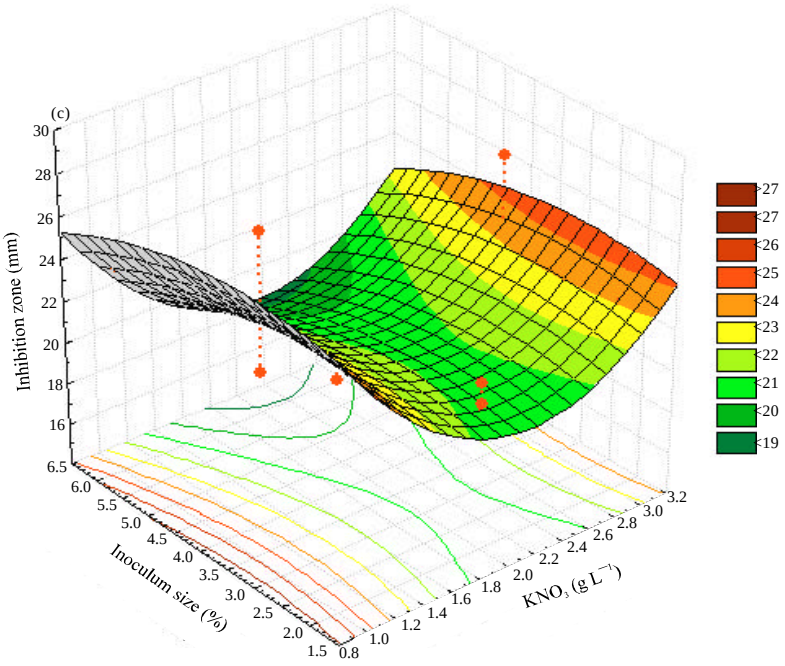

Fig. 5(a-c): Three-dimensional response surface plots showing the effect of: (a) KNO_3, (b) NaCl, and (c) Inoculum size and their mutual effect on the antimicrobial agent activity

vary. Figure 5a represents the antimicrobial agent activity as a function of $KNO_3(X_1)$, NaCl (X_2) by keeping inoculum size at optimum value. The highest value of antimicrobial agent activity was obtained with high level of KNO_3 and low level of NaCl, further increase of NaCl level did not result in higher antimicrobial agent yields. Figure 5b showed that the maximum activity found towards the center point of inoculum size and low value of NaCl. The maximum antimicrobial agent production was attained at low levels of both the factors, KNO_3 and inoculum size while further increase in the level of KNO_3 resulted in a gradual decrease in yield (Fig. 5c), further increase of inoculum size did not result in higher antimicrobial agent yields.

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 agent activity was 27 mm, where the predicted value from the polynomial model was 26.5 mm. The verification revealed a high degree of accuracy of the model of more than 98.15%, indicating the model validation under the tested conditions. The optimal levels of the process variables for antimicrobial agent production by *Streptomyces* sp. NEAE-1 were KNO_3 (3 g L^{-1}), NaCl (0.3 g L^{-1}) and inoculum size (4%, v/v)

CONCLUSION

A maximum extracellular antimicrobial agent production of 27 mm was achieved with the following optimized factors L^{-1}: starch 10 g, KNO_3 3 g, K_2HPO_4 0.5 g, NaCl 0.3 g, $MgSO_4.7H_2O$ 0.5 g, $CaCO_3$ 1 g, $FeSO_4.7H_2O$ 0.01 g, pH 6.5, temperature 25°C, agitation speed (200 rpm min^{-1}), medium volume 50 mL, inoculum size 4% (v/v), inoculum age 60 h and fermentation period 7 days. Validation experiments were also carried out to verify the adequacy and the accuracy of the model and results showed that the predicted values agreed with the experimental values well and more than one and half-fold increase compared to the original medium was obtained.

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