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Research Article
Antimicrobial Potentialities of *Streptomyces lienomycini* NEAE-31 Against Human Pathogen Multidrug-resistant *Pseudomonas aeruginosa*

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Abstract
Background and Objective: Antimicrobial drug resistance is one of the most serious problems because many bacteria that cause infections are becoming more resistant to the clinically available antibiotics already marketed. Thus, there is an urgent need to discover new antimicrobial agents effective against multi-resistant bacteria. **Materials and Methods:** The total of 130 morphologically different actinomycete strains were isolated from various soil samples collected from different regions of Egypt and Saudi Arabia and screened for their antimicrobial activities. *Streptomyces* sp. NEAE-31 was selected for more investigations and identified on the basis of morphological, cultural, physiological and biochemical properties, together with 16S rRNA sequence. Initial screening of fermentation parameters was performed using a Plackett-Burman design and the variables with statistically significant effects on the production of antimicrobial metabolites were identified. The most significant positive independent variables affecting bioactive metabolites production were selected for further optimization studies using face-centered central composite design. **Results:** The results indicated that *Streptomyces* sp. NEAE-31 exhibited a broad antimicrobial spectrum against several microorganisms including multidrug-resistant *Pseudomonas aeruginosa, E. coli, Staphylococcus aureus, Bacillus subtilis* and *Bipolaris oryzae*. *Streptomyces* sp. NEAE-31 was identified as *Streptomyces lienomycini* strain NEAE-31 and sequencing product was deposited in the GenBank database under accession number KF725623. Among the variables screened, yeast extract, $CaCO_3$ and inoculum size had positive significant effects on antimicrobial activities. The maximal antimicrobial activity is 41 mm inhibition zone. **Conclusion:** The statistical optimization resulted in about 1.64 fold increase in the production of bioactive metabolites by *Streptomyces lienomycini* strain NEAE-31. The results make this strain attractive for the pharmaceutical industry.

Key words: *Streptomyces*, isolation and identification, 16S rRNA, bioactive metabolites, optimization, multidrug-resistant *Pseudomonas aeruginosa*, Plackett-Burman design, face-centered central composite design

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa can cause infection of the blood, heart, central nervous system, ear, eyes, bones, joints, skin, urinary, gastrointestinal and the respiratory tract[1]. People with cystic fibrosis, diabetes, AIDS or cancer are especially at risk for infection with *Pseudomonas aeruginosa*. Patients in hospital are also very much at risk. *Pseudomonas* infection is potentially very serious and is often resistant to treatment, requiring two or more antibiotics, often intravenously.

The increase in the frequency of zmulti-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[2].

The actinomycetes are rich sources of a variety of bioactive products and these organisms have been famous as producers of secondary metabolites including antibacterial and antifungal antibiotics, anticancer drugs, natural herbicides and immunosuppressive agents[3]. It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes[4]. *Streptomyces* is the largest antibiotic-producing genus in the microbial world discovered so far. Recent reports show that this group of microorganisms still remains an important source of antibiotics[5].

To meet the growing demands in the industry it is necessary to improve the fermentation processes and thus increase the yield without increasing the cost of production[6]. Traditionally, fermentation processes have been optimized by changing one independent variable or factor at a time while keeping the others at some fixed values. The traditional optimization is slow and laborious, especially if a large number of independent variables are involved. Furthermore, it does not reflect the interaction effects among the variables employed[7]. Consequently, statistical optimization has become a common practice in biotechnology. It has the advantage of taking into account the interaction among factors on the outcome of the fermentation is less time consuming and avoids the erroneous interpretation occurring in one factor at a time optimization[8]. The Plackett-Burman design provides an efficient way of a large number of variables and identifying the most important ones[9]. Process optimization using Response Surface Methodology (RSM) usually involves simultaneous testing of many factors in a limited number of experiments. This method quantifies possible interactions between various factors, which is difficult to obtain using traditional optimization techniques[7].

The objectives of the present study were to isolate, screen bioactive metabolites producing actinomycetes and to identify the most active isolate. The optimization of physicochemical factors for bioactive metabolites production by *Streptomyces lienomycini* strain NEAE-31 was carried out in two steps: (1) Screening of the significant variables influencing bioactive metabolites production was carried out by 2-level factorial design using the Plackett-Burman design, (2) Face-centered central composite design was applied in the second step to determine the optimum levels of the factors that significantly influence the bioactive metabolites production.

MATERIALS AND METHODS

Microorganisms and cultural conditions: *Streptomyces* spp. used in this study were isolated from various soil samples collected from different localities of Egypt and Saudi Arabia. Actinomycetes from the soil had been isolated using standard dilution plate method procedure on petri plates containing starch nitrate agar medium 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 and agar 20 and distilled water up to 1 L, then plates were incubated for a period of 7 days at 30°C. Nystatin (50 µg mL^{-1}) was incorporated as an antifungal agent to minimize fungal contamination. The actinomycete strains predominant on media were picked out, purified and maintained on starch-nitrate agar slants. These strains were stored as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation. Biomass for chemotaxonomic and molecular systematic studies was obtained by growing the strain in shake flasks (at 200 rpm) using International *Streptomyces* Project (ISP) medium 2 broth[10] at 30°C for 2 days. Mycelia and cells were harvested by centrifugation, washed with distilled water and then freeze-dried.

Bioactive metabolites screening and selection of isolates: Bioactive metabolites 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 bioactive metabolites activities were also tested against a group of bacteria belonging to the Culture Collection of NRRL: Gram-positive (*Staphylococcus aureus* NRRL B-313 6538, *Bacillus subtilis* NRRL B-543), Gram-negative (*Escherichia coli* NRRL B-210, *Pseudomonas aeruginosa* NRRL B-23) and *Candida albicans* NRRL Y-477 and activities against 5 fungal strains (*Rhizoctonia solani*, *Alternaria solani*, *Bipolaris oryzae*, *Fusarium oxysporum* and *Fusarium solani*) was also determined.

Primary screening was carried out using the plug agar method. The actinomycetes isolates were lawn-cultured by dense streaking on starch nitrate medium plates and incubated at 30°C for 7 days. Nine millimeters agar discs were prepared using sterile cork borer from well grown culture and placed on fresh lawn culture of test organisms. The plates were then kept at 4°C for overnight for the diffusion of the antimicrobial metabolites and then incubated at 30°C. The zones of inhibition were determined after 24 h. Moreover, the active isolates obtained from the primary screening were subjected to secondary screening by agar well diffusion method. Erlenmeyer flasks (250 mL) containing 50 mL of starch nitrate broth were inoculated with three discs of 7 days old plate culture and incubated at 30°C for 5 days at 150 rpm. The culture broth was centrifuged and the activity of the supernatant was determined against test organisms by adding 100 μL to wells (9 mm) bored into freshly inoculated plates. The plates were then kept at 4°C for 3 h for diffusion of the antimicrobial metabolites; they were then incubated at 30°C. The zones of inhibition were determined after 24 h.

Morphology and cultural characteristics: The morphology of the spore chain and the spore surface ornamentation of strain NEAE-31 were examined on starch nitrate agar medium after 14 days of incubation at 30°C. The gold-coated dehydrated specimen can be examined at different magnifications with analytical scanning electron microscope Jeol JSM-6360 LA operating at 20 kV at the Central Laboratory, City for Scientific Research and Technology Applications, Alexandria, Egypt. Aerial spore-mass colour, substrate mycelial pigmentation and the production of diffusible pigments were observed on yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt starch agar (ISP medium 4), glycerol-asparagine agar (ISP medium 5) peptone-yeast extract iron agar (ISP medium 6), tyrosine agar (ISP medium 7) as described by Shirling and Gottlieb[10]; all plates were incubated at 30°C for 14 days.

Physiological characteristics: Strain NEA-31 was examined for biochemical and physiological characteristics according to the established methods described by Williams *et al.*[11] and Kampfer *et al.*[12] and following the guidelines of the International *Streptomyces* Project (ISP)[10,13].

16S rRNA sequencing: The DNA was isolated by the method of Sambrook *et al.*[14]. The PCR amplification reaction was performed in a total volume of 100 μL, which contained 1 μL DNA, 10 μL of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 μL PCR buffer, 3.5 μL 25 mM $MgCl_2$ and 0.5 μL Taq polymerase, 4 μL of 10 pmol (each) forward 16S rDNA primer 27 f (5'-AGAGTTTGATCMTGCCTCAG-3') and reverse 16S rDNA primer 1492 r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 μL. Amplification was carried out with an initial incubation of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, followed by a 10 min final extension at 72°C. The PCR product was purified with a QIA quick PCR purification kit (Qiagen). Amplified product was sequenced directly on a 3100 automatic DNA sequencer (Applied Biosystems) and deposited in the GenBank database under accession number KF725623.

Sequence alignment and phylogenetic analysis: The partial 16S rRNA gene sequence of strain NEAE-31 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih.gov/blst)[15] and the software package[16] MEGA4 version 2.1 was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the neighbor-joining algorithm[17] based on the 16S rRNA gene sequences of strain NEAE-31 and related organisms.

Inoculum preparation: About 250 mL Erlenmeyer flasks containing 50 mL of yeast-malt extract broth (malt extract 1%, dextrose 0.4%, yeast extract 0.4%, agar 2% at pH 7.0) were inoculated with three disks of 9 mm diameter taken from the 7 days old stock culture grown starch nitrate agar medium. The flasks were incubated for 24-48 h in a rotatory incubator shaker at 30°C and 200 rpm and were used as inoculum for subsequent experiments.

Bioactive metabolites production conditions: Fifty millimeter 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 desired conditions. After the specified incubation time for each set of experimental trials, the mycelium of the isolate was collected by centrifugation at 5000 rpm for 15 min. The cell free supernatant was used for bioactive metabolites activities determinations.

Antagonistic action against microbial test strains: This was carried out using the well-diffusion technique, nutrient agar as an assay medium and *Pseudomonas aeruginosa* as a test organism. The nutrient agar was poured into sterile petri-dishes and allowed to solidify. After solidifying, plates were inoculated with 0.1 mL suspension of the test strain and wells were punched out using 9 mm cork borer. One hundred microliter of tested filtrates was transferred into each well. Petri-dishes were kept in a refrigerator for 3 h to allow for the diffusion of the bioactive metabolites. Petri-dishes were then incubated inverted for 24 h at 30°C. The inhibition zone diameter was measured in millimeter.

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 bioactive 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[18]. The total number of experiments to be carried out according to Plackett-Burman is n+1, where n is the number of variables[19]. Each variable is represented at two levels, high and low denoted by (+) and (–), respectively. Table 1 shows the 15 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, fermentation time and inoculum age were 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 variable (bioactive 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.

Face-centered central composite design (FCCD): This step involved optimization of the levels and the interaction effects between various significant variables which exerted a positive effect on the bioactive metabolites activity by using face-centered central composite design (FCCD). The FCCD is an effective design that is used for sequential experimentation and provides reasonable amount of information for testing the goodness of fit and does not require large number of design points thereby reducing the overall cost associated with the experiment[20]. In this study, the experimental plan consisted of 20 trials and the independent variables were studied at three different levels, low (-1), middle (0) and high (+1). The center point was repeated six times in order to evaluate the curvature and the experiment replication facilitated the pure error estimation, so that the significant lack of fit of the models could be predicted. All the experiments were done in duplicate and the average of bioactive metabolites activity obtained was taken as the dependent variable or response (Y). The experimental results of FCCD 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 metabolites by *Streptomyces lienomycini* NEAE-31 using Plackett-Burman design

		Levels	
Codes	Independent variables	-1	+1
A	Starch (g L^{-1})	10	20
B	KNO_3 (g L^{-1})	1	2
C	K_2HPO_4 (g L^{-1})	0.5	1
D	Yeast extract (g L^{-1})	0	0.1
E	NaCl (g L^{-1})	0.1	0.5
F	$MgSO_4.7H_2O$ (g L^{-1})	0.1	0.5
G	$CaCO_3$ (g L^{-1})	1	3
H	$FeSO_4$ (g L^{-1})	0.01	0.02
J	pH	7	9
K	Temperature (°C)	30	37
L	Agitation speed (rpm)	150	200
M	Medium volume (mL/250 mL flask)	50	75
N	Inoculum size (% v/v)	2	4
O	Fermentation time (days)	5	7
P	Inoculum age (h)	24	48

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

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.

Statistical analysis: Design Expert® 7.0 software version 7 (Stat-Ease Inc., USA) for windows was used for the experimental designs and statistical analysis. The statistical software package, STATISTICA software (Version 8.0, StatSoft Inc., Tulsa, USA) was used to plot the three-dimensional surface plots.

RESULTS AND DISCUSSION

Isolation and screening: The total of 130 morphologically different actinomycete strains were isolated from soil samples from different regions of Egypt and Saudi Arabia. All these isolates were purified and screened for their bioactive metabolites activity. Out of these, 15% of the isolates exhibited bioactive metabolites activity during the preliminary screening experiment. On the basis of larger clear zones against multidrug-resistant *Pseudomonas aeruginosa*, one strain (*Streptomyces* sp. NEAE-31) was chosen and the optimization of bioactive metabolites production was performed on the selected strain.

Bioactive metabolites activity: The activity of secondary metabolites was tested against Gram-negative, Gram-positive bacteria and fungal test organisms (Table 2). The results showed that *Streptomyces* sp. NEAE-31 exhibited interesting bioactive metabolites activities. There was a strong activity against *Pseudomonas aeruginosa* NRRL B-23, multidrug resistant *Pseudomonas aeruginosa* T9934. Moreover, the halo diameter obtained with *Pseudomonas aeruginosa* NRRL B-23, multidrug resistant *Pseudomonas aeruginosa* T9934, *Escherichia coli* NRRL B-210, multidrug resistant *Staphylococcus aureus* A9897, *Staphylococcus aureus* NRRL B-313, *Bacillus subtilis* NRRL B-543 were 27, 25, 19, 20, 22 and 23 mm respectively. There is no activity against multidrug resistant *Klebsiella pneumonia* A9898. The halo diameter obtained with *Bipolaris oryzae* was 21 mm while there is no activity against *Rhizoctonia solani*, *Alternaria solani*, *Fusarium oxysporum* and *Fusarium solani*.

Morphology and cultural characteristics of the isolate NEAE-31: Aerial mycelium and substrate mycelium were well-developed without fragmentation. Aerial mycelia are yellow on most agar media and the color of the substrate mycelium was varied from yellow to orange (Fig. 1a, Table 3). A scanning electron micrograph of spore chains of strain NEAE-31 cultured on starch nitrate agar medium revealed that the spore chains were rectiflexibles and the spores were short rods to oval in shape with a smooth surface (Fig. 2). Yellow diffusible pigments are produced on most media. Strain NEAE-31 grew well on yeast extract/malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salts/starch agar (ISP medium 4) and tyrosine agar (ISP medium 7) but weak growth was observed on glycerol-asparagine agar (ISP medium 5) and peptone-yeast extract iron agar (ISP medium 6).

Physiological characteristics of the isolate NEAE-31: The physiological and biochemical characteristics of strain NEAE-31 are shown in Table 4. Melanoid pigments not formed in Tryptone-yeast extract broth (ISP medium 1), peptone-yeast extract iron agar (ISP medium 6) or tyrosine agar (ISP medium 7). As the sole carbon source, it utilizes D-xylose, D-glucose, D (+) mannose, sucrose, D-galactose, cellulose, rhamnose, raffinose, α-lactose, ribose and D-maltose for growth. Some growth occurs with D-fructose and L-arabinose as the carbon source and there is no growth on gluconic acid. Degrades casein, gelatin, starch and cellulose.

Table 2: Antimicrobial activity of the antimicrobial metabolites produced by *Streptomyces lienomycini* NEAE-31

Microorganisms	Specification	Inhibition zone diameter (mm)
Gram negative bacteria		
Pseudomonas aeruginosa	NRRL B-23 resistant to ceftriaxone,	27
Pseudomonas aeruginosa T9934	Gentamicin, cefotaxime, trimethoprim-sulfamethoxazole and augmentin	25
Escherichia coli	NRRL B-210	19
Klebsiella pneumonia A9898	Resistant to trimethoprim-sulfamethoxazole, augmentin, gentamicin, ceftriaxone, amikacin and cefotaxime	Negative
Gram positive bacteria		
Staphylococcus aureus A9897	Resistant to vancomycin, augmentin, gentamicin, trimethoprim-sulfamethoxazole, oxacillin, amikacin and tobramycin	20
Staphylococcus aureus	NRRL B-313	22
Bacillus subtilis	NRRL B-543	23
Fungi		
Bipolaris oryzae		21
Fusarium oxysporum		Negative
Fusarium solani		Negative
Rhizoctonia solani		Negative
Alternaria solani		Negative

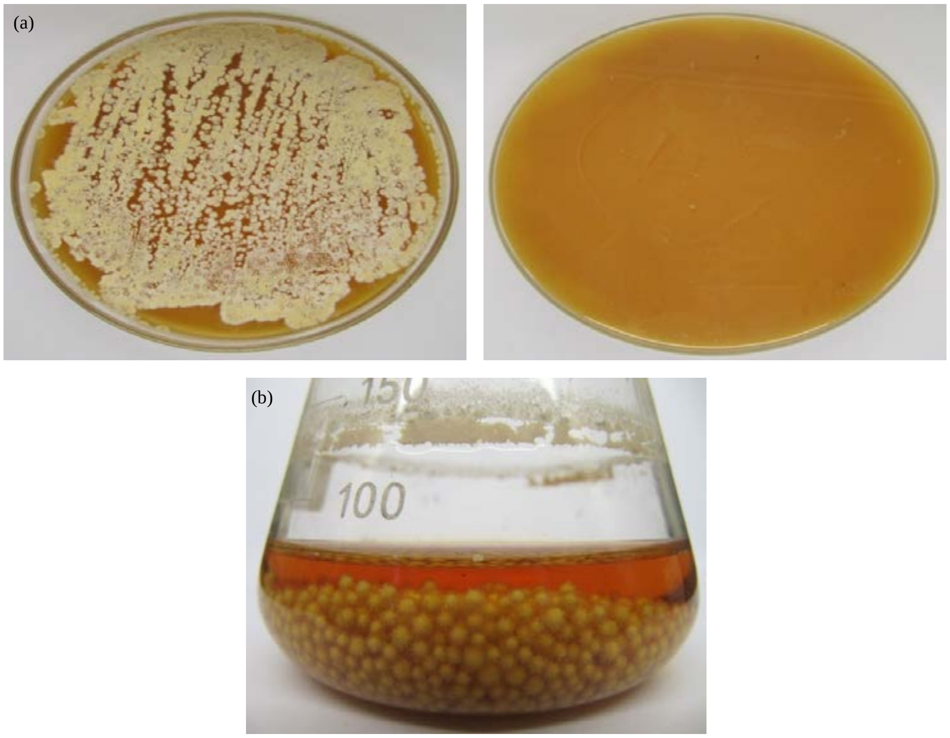

Fig. 1(a-b): (a) Color of the aerial and substrate mycelium of *Streptomyces lienomycini* NEAE-31 strain grown on starch-nitrate agar medium for 14 days of incubation at 30°C and (b) Growth of *Streptomyces lienomycini* NEAE-31 in large, yellow spherical pellets form during the antimicrobial metabolites production in shake flasks after inoculation and incubation on a rotary shaker (200 rpm) at 30°C

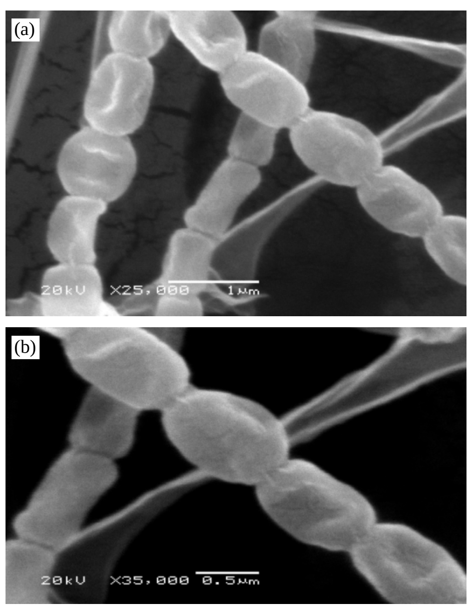

Fig. 2(a-b): Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of strain NEAE-31 grown on inorganic salts/starch agar medium for 14 days at 30°C at magnification of (a) 25000X and (b) 35000X

Fig. 3: Plate assay showing zone of hydrolysis of starch by strain NEAE-31. All the starch in the medium near the microbe has been hydrolyzed by extracellular amylases

Table 3: Culture characteristics of the *Streptomyces* isolate NEAE-31

Medium	Aerial mycelium	Substrate mycelium	Diffusible pigment	Growth
ISP medium 2 (Yeast extract-malt extract agar)	Yellow	Orange	Yellow	Excellent
ISP medium 3 (Oatmeal agar)	Yellow	Yellow	Yellow	Excellent
ISP medium 4 (Inorganic salt-starch agar)	Yellow	Yellow	Yellow	Excellent
ISP medium 5 (Glycerol asparagines agar)	Faint yellow	Yellow	Faint yellow	Weak
ISP medium 6 (Peptone-yeast extract iron agar)	No-sporulation	No-sporulation	Non-pigmented	Weak
ISP medium 7 (Tyrosine agar)	Yellow	Orange	Yellow	Excellent

Gelatin liquefaction, starch hydrolysis (Fig. 3), milk coagulation and peptonization are positive whereas reductions of nitrate to nitrites are negative. Protease, cellulase and chitosanase activities are positive whereas L-asparaginase and lecithinase activities are negative. Uricase activity is doubtful. Growth occurs in the presence of NaCl up to 5% w/v. Strain NEAE-31 exhibited interesting bioactive metabolites activities against *Pseudomonas aeruginosa* NRRL B-23, multidrug resistant *Pseudomonas aeruginosa* T9934, *Escherichia coli* NRRL B-210, multidrug resistant *Staphylococcus aureus* A9897, *Staphylococcus aureus* NRRL B-313, *Bacillus subtilis* NRRL B-543 and *Bipolaris oryzae*. There is no activity against multidrug resistant *Klebsiella pneumonia* A9898, *Rhizoctonia solani*, *Alternaria solani*, *Fusarium oxysporum* and *Fusarium solani.*

Molecular phylogeny of the isolate NEAE-31: The 16S rRNA gene sequence (1432 bp) of strain NEAE-31 was deposited in the GenBank database under the accession number KF725623. The 16S rRNA gene sequence of strain NEAE-31 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST[15]. The phylogenetic tree based on 16S rRNA gene sequence of strain NEAE-31 and most closely related type strains of species of the genus *Streptomyces* (Fig. 4) showed that the isolate falls into one distinct subclade with *Streptomyces lienomycini* strain 173894 (GenBank accession No. EU570419.1) with which it shared 16S rRNA gene sequence similarity of 98.0%. It is clear that the strain NEAE-31 is closely similar to *Streptomyces lienomycini.* Thus, it was given the suggested name *Streptomyces lienomycini* NEAE-31.

Evaluation of the factors affecting bioactive metabolites activity using Plackett-Burman design: A total of 15 independent (assigned) and four unassigned variables (commonly referred as dummy variables) were screened in Plackett-Burman experimental design. Dummy variables (D_1, D_2, D_3 and D_4) are used to estimate experimental errors in data analysis. The experiment was conducted in 20 runs to study the effect of the selected variables on the production of bioactive metabolites (Table 5). All trials were performed in duplicate and the average of bioactive metabolites production (inhibition zone (mm)) were treated as responses. Plackett-Burman statistical design is a well-established and suitable for complicated systems with multiple variables to screen out and select most significant environmental and nutritional variables. Compared with other medium design strategies, the Plackett-Burman design is simple and fast method for

Table 4: Physiological and biochemical characteristics of the *Streptomyces* NEAE-31

Characteristics	*Streptomyces* NEAE-31
Aerial mycelium on ISP medium 2	Yellow
Substrate mycelium on ISP medium 2	Orange
Production of diffusible pigment	Yellow
Spore chain morphology	Rectiflexibles
Spore surface	Smooth
Spore shape	Elongated
Sensitivity of diffusible pigment to pH	+
Melanin production on	
Tryptone-yeast extract broth (ISP medium 1)	-
Peptone-yeast extract iron agar (ISP medium 6)	-
Tyrosine agar (ISP medium 7)	-
Degradation of	
Casein	+
Gelatin	+
Starch	+
Cellulose	+
Max NaCl tolerance (% w/v)	5%
Growth on sole carbon source (1% w/v) utilization of carbon sources	
D (-) fructose	±
D (+) xylose	+
D (+) glucose	+
D (+) mannose	+
Sucrose	+
D (+) galactose	+
Cellulose	+
Rhamnose	+
Raffinose	+
L-arabinose	±
Gluconic acid	-
α-lactose	+
Ribose	+
D-maltose	+
Enzymes	
α-amylase (starch hydrolysis)	+
Gelatinase (gelatin liquification)	+
Protease	+
Cellulase	+
Uricase	±
Chitosanase	+
L-asparaginase	-
Lecithinase activity	-
Reduction of nitrates to nitrite	-
Coaggulation of milk	+
Peptonization of milk	+
Antimicrobial activities	
Pseudomonas aeruginosa NRRL B-23	+
Multidrug resistant *Pseudomonas aeruginosa* T9934	+
Pseudomonas aeruginosa NRRL B-23	+
Escherichia coli NRRL B-210	+
Multidrug resistant *Staphylococcus aureus* A9897	+
Staphylococcus aureus NRRL B-313	+
Bacillus subtilis NRRL B-543	+
Bipolaris oryzae	+
Klebsiella pneumonia A9898	-
Rhizoctonia solani	-
Alternaria solani	-
Fusarium oxysporum	-
Fusarium solani	-

+: Positive, -: Negative, ±: Doubtful

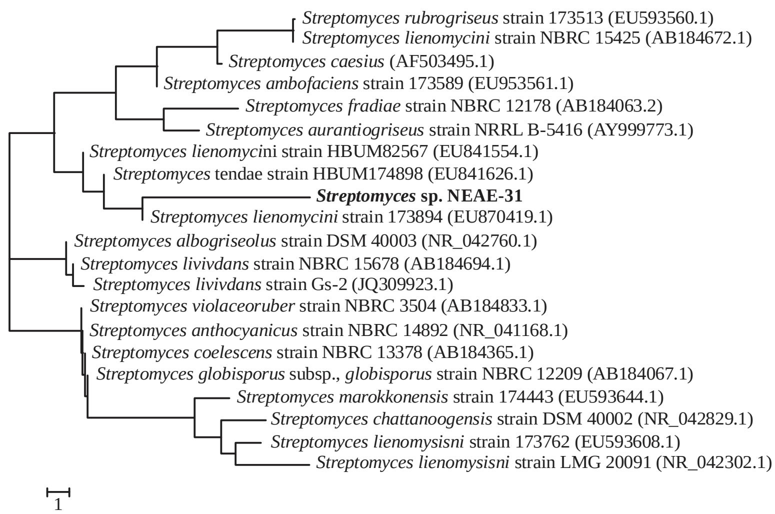

Fig. 4: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain NEAE-31 and related species of the genus *Streptomyces*. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA4

Table 5: Twenty-trial Plackett-Burman experimental design for evaluation of 19 independent variables with coded values along with the observed antimicrobial metabolites activity produced by *Streptomyces lienomycini* strain NEAE-31

	Coded levels of independent variables																			Inhibition zone (mm)		
Run order	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	D_1	D_2	D_3	D_4	Actual value	Predicted value	Residuals
1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	24	23.9	0.1
2	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	37	36.9	0.1
3	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	30	30.1	-0.1
4	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	26	25.9	0.1
5	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	31	30.9	0.1
6	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	34	34.1	-0.1
7	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	29	29.1	-0.1
8	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	31	30.9	0.1
9	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	20	19.9	0.1
10	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	19	18.9	0.1
11	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	19	18.9	0.1
12	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	27	27.1	-0.1
13	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	23	22.9	0.1
14	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	33	33.1	-0.1
15	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	35	34.9	0.1
16	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	17	17.1	-0.1
17	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	22	22.1	-0.1
18	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	20	20.1	-0.1
19	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	23	23.1	-0.1
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	28	28.1	-0.1

X_1-X_{15}: Independent (assigned) variables, D_1-D_4: Dummy variables (unassigned), 1: High level of variables, -1: Low level of variables

screening large number of variables in one experiment to see which gives the best results and is often used to evaluate the important variables affecting culture requirements.

Streptomyces lienomycini NEAE-31 growth has been shown as large spiny yellow spherical pellets (Fig. 1b) 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[21]. Plackett-Burman experiments showed a markedly wide variation (17-37 mm) in inhibition zone; this variation reflected the importance of medium optimization to attain higher bioactive metabolites production. The maximum inhibition zone (37 mm) was achieved in the run number 2, while the minimum inhibition zone (17 mm) was observed in the run number 16.

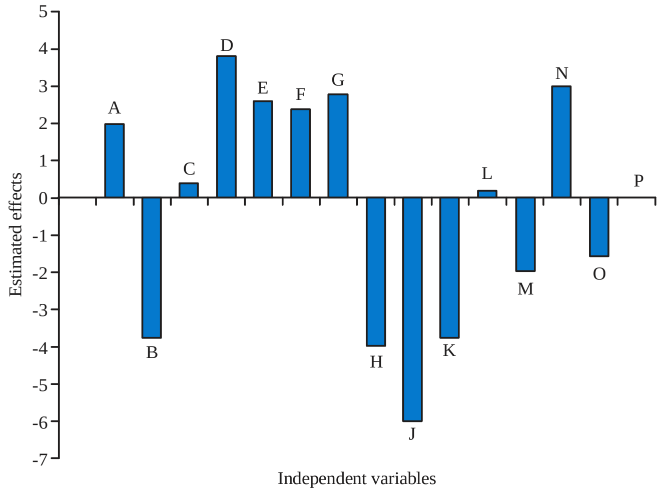

Fig. 5: Main effects of the factors affecting bioactive metabolites production according to the Plackett-Burman experimental results

Table 6: Regression coefficients, estimated effect and percentage of contribution for antimicrobial metabolites production by *Streptomyces lienomycini* strain NEAE-31 using Plackett-Burman design

Terms	Coefficient	Effect	Contribution (%)
Intercept	26.4	52.8	
A-starch	1.0	2.0	5.21
B-KNO_3	-1.9	-3.8	9.90
C-K_2HPO_4	0.2	0.4	1.04
D-yeast extract	1.9	3.8	9.90
E-NaCl	1.3	2.6	6.77
F-$MgSO_4.7H_2O$	1.2	2.4	6.25
G-$CaCO_3$	1.4	2.8	7.29
H-$FeSO_4.7H_2O$	-2.0	-4.0	10.42
J-pH	-3.0	-6.0	15.63
K-temperature	-1.9	-3.8	9.90
L-agitation	0.1	0.2	0.52
M-medium volume	-1.0	-2.0	5.21
N-inoculum size	1.5	3.0	7.81
O-fermentation time	-0.8	-1.6	4.17

Table 7: Regression statistics and analysis of variance (ANOVA) for the experimental results of Plackett-Burman design used for antimicrobial metabolites production by *Streptomyces lienomycini* strain NEAE-31

Sources	SS	MS	F-value	p>F	Confidence level (%)
Model	680.6	40.03529	400.35	0.0025*	99.75
A	20	20	200	0.0050*	99.5
B	72.2	72.2	722	0.0014*	99.86
C	0.8	0.8	8	0.1056	89.44
D	72.2	72.2	722	0.0014*	99.86
E	33.8	33.8	338	0.0029*	99.71
F	28.8	28.8	288	0.0035*	99.65
G	39.2	39.2	392	0.0025*	99.75
H	80	80	800	0.0012*	99.88
J	180	180	1800	0.0006*	99.94
K	72.2	72.2	722	0.0014*	99.86
L	0.2	0.2	2	0.2929	70.71
M	20	20	200	0.0050*	99.5
N	45	45	450	0.0022*	99.78
O	12.8	12.8	128	0.0077*	99.23
Residual	0.2				
Cor total	680.8				

SD	0.3162	R-squared	0.9997
Mean	26.4	Adj R-squared	0.9972
CV (%)	1.1978	Pred R-squared	0.9706
PRESS	20	Adeq precision	66

*Significant values, SS: Sum of squares, MS: Mean square, F: Fishers's function, P: Level of significance, PRESS: Predicted residual sum of squares, CV%: Coefficient of variation %, SD: Standard deviation

The relationship between a set of independent variables and the response (Y) is determined by a mathematical model called multiple-regression model. Statistical analysis of the responses were performed which is represented in Table 6 and 7. The data revealed that, inoculum age (P) is insignificant variable with 0 effect (0.0) and 0% of contribution (0.0). Lower percentage of contribution indicated higher p-value. Thus instead of starting with the maximum model effects, backward regression at α 0.15 was applied to eliminate the effect of inoculum age (P). Then, the model fitted for the test of significance. Table 6 and Fig. 5 show the main effect of each variable on the bioactive metabolites production. Main effect allows the determination of the effect of each variable. A large effect either positive or negative indicates that a factor has a large impact on production, while an effect close to 0 means that a factor has little or no effect. With respect to the main effect of each variable, it can seen that 8 variables from the 15 named starch, K_2HPO_4, yeast extract, NaCl, $MgSO_4.7H_2O$, $CaCO_3$, agitation speed and inoculum size affect positively on bioactive metabolites production, where sex variables named (KNO_3, $FeSO_4.7H_2O$, pH, temperature, medium volume and fermentation time) affect negatively on bioactive metabolites production and

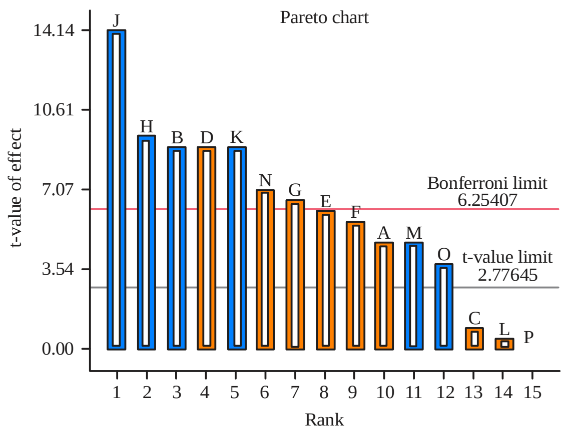

Fig. 6: Pareto chart shows the amount of influence of each factor on the bioactive metabolites production according to the Plackett-Burman experimental results

inoculum age has no effect on bioactive metabolites production. The significant variables with positive effect were fixed at high level and the variables which exerted a negative effect on bioactive metabolites production were maintained at low level for further optimization by face-centered central composite design. The percentages contributions of the variables are given in Table 6. The results revealed that pH, $FeSO_4.7H_2O$, KNO_3, yeast extract, temperature, inoculum size and $CaCO_3$ are the most contributing components with 15.63, 10.42, 9.90, 9.90, 9.90, 7.81 and 7.29%, respectively. Also, it was clear that among the seven variables, only yeast extract, inoculum size and $CaCO_3$ exerted positive effects, whereas the other variables (pH, $FeSO_4.7H_2O$, KNO_3, temperature) exerted a negative effects on bioactive metabolites production, which means that the increase in the yeast extract, inoculum size and $CaCO_3$ and decrease in pH, $FeSO_4.7H_2O$, KNO_3, temperature could exert positive effect on bioactive metabolites production.

The Pareto chart illustrates the order of effects of the variables affecting bioactive metabolites production in Plackett-Burman experimental design (Fig. 6). It displays the absolute values of the effects and draws a reference line on the chart. Any effect that extends past this reference line is potentially important. Pareto chart in design expert version 7.0 reproduce the relation between t-value (effect) vs. ranks. Among the 15 assigned variables, pH was the most significant variable affecting bioactive metabolites production at 99.94% confidence followed by $FeSO_4.7H_2O$ at 99.88% confidence.

The analysis of variance (ANOVA) of the experimental design was calculated and the sum of square, mean square, F-value, p-value and confidence level are given in Table 7. The significance of the model was calculated by the p-value. The p-value is the probability which serves as a tool for checking the significance of each of the parameter. The model F-value of 400.35 and p-value (0.0025) implies that the model is significant. There is only a 0.25% chance that a "Model F-value" this large could occur due to noise. Values of "Prob> F" (p-value) less than 0.05 indicate model terms are significant. The data revealed that, 12 variables (starch, KNO_3, yeast extract, NaCl, $MgSO_4$, $CaCO_3$, $FeSO_4$, pH, temperature, medium volume, inoculum size and fermentation time) were found to significantly affect bioactive metabolites production while the three variables (K_2HPO_4, agitation speed and inoculum age) have not significant influence on the bioactive metabolites production (Table 7). The analysis showed that, pH (J) with a probability value of 0.0006 was determined to be the most significant factor affecting bioactive metabolites production by *Streptomyces lienomycini* NEAE-31 at 99.94% confidence followed by $FeSO_4.7H_2O$ (H) ($p = 0.0012$) at 99.88% confidence and KNO_3 (B), yeast extract (D), temperature (K) ($p = 0.0014$) at 99.86% confidence, then inoculum size (N) ($p = 0.0022$) at 99.78% confidence and $CaCO_3$ (G) ($p = 0.0025$) at 99.75% confidence. The lower probability values indicate the more significant factors affecting bioactive metabolites production.

The R^2 values provide a measure of how much variability in the experimental 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[22]. The value of the determination coefficient (R^2) was found to be 0.9997 indicates that 99.97% of the variability in the bioactive metabolites production could be explained by the model independent variables and only 0.03% of the total variations are not explained by the independent variables. The adjusted R^2 (0.9972) is also very high that indicates that the model is very significant. The "Pred R-squared" of 0.9706 is in reasonable agreement with the "Adj R-squared" of 0.9972. This indicated a good adjustment between the observed and predicted values. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 66 indicates an adequate signal.

The coefficient of variation percentage (CV%) is a measure of residual variation of the data relative to the size of the mean. Usually, the higher the value of CV, the lower is the reliability of experiment. Here, a lower value of CV (1.1978%) indicates a greater reliability of the experimental performance. The predicted residual sum of squares (PRESS) is a measure of how well the model fits each point in the design. The smaller the PRESS statistic, the better the model fits the data points. Our value of PRESS is 20. The model shows standard deviation and mean value of 0.3162 and 26.4, respectively.

Table 8: Face-centered central composite design representing the antimicrobial metabolites production by *Streptomyces lienomycini* strain NEAE-31 as influenced by yeast extract (X_1), $CaCO_3$ (X_2) and inoculum size (X_3) along with the predicted antimicrobial metabolites activity and residuals and the levels of variables with actual factor levels corresponding to coded factor levels

		Variables			Inhibition zone (mm)		
Standard order	Run order	X_1	X_2	X_3	Experimental	Predicted	Residuals
16	1	0	0	0	41	40	1
11	2	0	-1	0	31	34	-3
8	3	1	1	1	30	30	0
6	4	1	-1	1	35	35	0
14	5	0	0	1	36	37	-1
13	6	0	0	-1	29	31	-2
17	7	0	0	0	41	40	1
20	8	0	0	0	41	40	1
15	9	0	0	0	41	40	1
19	10	0	0	0	41	40	1
9	11	-1	0	0	34	35	-1
10	12	1	0	0	39	41	-2
3	13	-1	1	-1	23	23	0
1	14	-1	-1	-1	19	18	1
7	15	-1	1	1	20	21	-1
2	16	1	-1	-1	22	21	1
18	17	0	0	0	41	40	1
4	18	1	1	-1	27	27	0
12	19	0	1	0	34	34	0
5	20	-1	-1	1	28	27	1

Level	Yeast extract (g L^{-1})	$CaCO_3$ (g L^{-1})	Inoculum size (% v/v)
-1	0.1	2	2
0	0.2	3	4
1	0.5	4	6

The first order polynomial equation was derived representing bioactive metabolites production as a function of the independent variables to approach the optimum response. By neglecting the insignificant terms the following regression equation in terms of coded factors was obtained:

$$Y_{(\text{bioactive metabolites production})} = 26.4+1A-1.9B+1.9D+1.3E+1.2F+1.4G-2H-3J-1.9K-1M+1.5N-0.8O \quad (3)$$

where, Y is the response and A, B, D, E, F, G, H, J, K, M, N and O are starch, KNO_3, yeast extract, NaCl, $MgSO_4$, $CaCO_3$, $FeSO_4$, pH, temperature, medium volume, inoculum size and fermentation time, respectively. The coefficient of each variable represents the effect extent of these variables on bioactive metabolites production.

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 20, KNO_3 1, K_2HPO_4 1, yeast extract 0.1, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 3, $FeSO_4.7H_2O$ 0.01, pH 7, temperature 30°C, agitation of 200 rpm, medium volume 50 mL, inoculum size 4 mL, fermentation time 5 days and inoculum age 24 h gives (36 mm) which is higher than result obtained from the basal medium before applying Plackett-Burman by 1.44 times (25 mm).

Optimization by face-centered central composite design: The face-centered central composite design was employed to study the interactions among the significant variables and also determine their optimal levels. Results of Placket-Burman design revealed that yeast extract, $CaCO_3$ and inoculum size were the most significant positive independent variables affecting bioactive metabolites production, thus they were selected for further optimization using face-centered central composite design. In this study, a total of 20 experiments with different combination of yeast extract (X_1), $CaCO_3$ (X_2) and inoculum size (X_3) were performed and the results of experiments are presented along with predicted response and residuals. Concentrations of three independent variables at different coded and actual levels of the variables also presented in Table 8. The central point was repeated six times (run order: 1, 7, 8, 9, 10 and 17). The results show considerable variation in the bioactive metabolites production. The maximum bioactive metabolites production (41 mm) was achieved in runs number run order 1, 7, 8, 9, 10 and 17 under the conditions of yeast extract (0.2 g L^{-1}), $CaCO_3$ (3 g L^{-1}) and inoculum size (4% v/v), while the minimum bioactive metabolites production (19 mm) was observed in run number 14 under the conditions of yeast extract (0.1 g L^{-1}), $CaCO_3$ (2 g L^{-1}) and inoculum size (2% v/v).

Table 9: Regression statistics of FCCD for optimization of antimicrobial metabolites production by *Streptomyces lienomycini* strain NEAE-31

Factor	Coefficient estimate	Standard error	95% CI low	95% CI high
Intercept	40.13	0.53	38.94	41.31
X_1	2.90	0.49	1.81	3.99
X_2	-0.10	0.49	-1.19	0.99
X_3	2.90	0.49	1.81	3.99
X_1X_2	0.50	0.55	-0.72	1.72
X_1X_3	1.25	0.55	0.03	2.47
X_2X_3	-2.75	0.55	-3.97	-1.53
X_1^2	-2.32	0.93	-4.40	-0.24
X_2^2	-6.32	0.93	-8.40	-4.24
X_3^2	-6.32	0.93	-8.40	-4.24
SD	1.546	R-squared	0.9784	
Mean	32.650	Adj R-squared	0.9590	
CV (%)	4.736	Pred R-squared	0.8764	
PRESS	137.043	Adeq precision	20.3375	

CV: Coefficient of variation, SD: Standard deviation

Multiple regression analysis and ANOVA: The data were analyzed using Design Expert® 7.0 for Windows to perform statistical analysis. The positive coefficients for X_1, X_3, X_1X_2, X_1X_3 (Table 9) indicate that linear effect of X_1, X_3 and interaction effects for X_1X_2, X_1X_3 increase bioactive metabolites production, while other negative coefficients indicate decrease in bioactive metabolites production. Value of PRESS is 137.043. The model shows standard deviation and mean value of 1.546 and 32.65, respectively (Table 9). In the present case, a lower value of C.V. (4.736) indicated a better precision and reliability of the experimental performance[23]. The determination coefficient (R^2) of the model was 0.9784 (Table 9) indicating that 97.84% of variability in the bioactive metabolites production was attributed to the independent variables and only 2.06% 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[24]. 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 bioactive metabolites production in the present study. The highest R^2 value showed the good agreement between the experimental results and the theoretical values predicted by the model[25]. The "Pred R-squared" of 0.8764 is in reasonable agreement with the "Adj R-squared" of 0.9590. This indicated a good adjustment between the observed and predicted values. "Adeq precision" ratio of 20.337 indicates an adequate signal to noise ratio. This model can be used to navigate the design space.

In order to evaluate the relationship between dependent and independent variables and to determine the maximum bioactive metabolites production corresponding to the optimum levels of yeast extract (X_1), $CaCO_3$ (X_2) and inoculum size (X_3), a second-order polynomial model (Eq. 4) 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:

$$Y_{(\text{Bioactive metabolites activity})} = 40.13+2.90\,X_1-0.10\,X_2+2.90\,X_3+0.5\,X_1X_2 +1.25\,X_1X_3-2.75\,X_2X_3-2.32X_1^2-6.32X_2^2-6.32X_3^2.... \quad (4)$$

where, Y is the response (bioactive metabolites activity) and X_1, X_2 and X_3 are yeast extract, $CaCO_3$ and inoculum size, respectively.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results are shown in Table 10. The model F-value of 50.41 with a very low probability value (p model> F 0.0001) implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. It can be seen from the degree of significance that the linear coefficients of yeast extract (X_1), inoculum size (X_3), interaction between yeast extract (X_1), inoculum size (X_3), interaction between $CaCO_3$ (X_2), inoculum size (X_3) and quadratic effect of yeast extract (X_1), $CaCO_3$ (X_2) and inoculum size (X_3) are significant model terms. The probability values of the coefficient suggest that among the three variables studied, X_2, X_3 shows maximum interaction between the two variables (p = 0.0005), indicating that 99.95% of the model affected by these variables. On the other hand, the linear coefficients of $CaCO_3$ (X_2) is not significant. Furthermore, among the different interactions, interaction between X_1 and X_2 is not significant (p = 0.3819), indicating that there is no significant correlation between each two variables and that they did not help much in increasing the production of bioactive metabolites.

Table 10: Analysis of variance (ANOVA) for FCCD results used for optimizing antimicrobial metabolites production by *Streptomyces lienomycini* strain NEAE-31

Sources	Sum of squares	dF	Mean square	F-value	p>F
Model	1084.64	9	120.52	50.41	<0.0001*
X_1	84.10	1	84.10	35.17	0.0001*
X_2	0.10	1	0.10	0.04	0.8421
X_3	84.10	1	84.10	35.17	0.0001*
X_1X_2	2.00	1	2.00	0.84	0.3819
X_1X_3	12.50	1	12.50	5.23	0.0453*
X_2X_3	60.50	1	60.50	25.30	0.0005*
X_1^2	14.78	1	14.78	6.18	0.0322*
X_2^2	109.78	1	109.78	45.91	<0.0001*
X_3^2	109.78	1	109.78	45.91	<0.0001*
Residual	23.91	10	2.39		
Lack of fit	23.91	5	4.78		
Pure error	0.00	5	0		
Cor total	1108.55	19			

*Significant values, df: Degree of freedom, F: Fishers's function, p: Level of significance

Table 11: Fit summary for experimental data

Sequential model sum of squares					
Sources	Sum of squares	df	Mean square	F-value	p>F
Linear vs mean	168.3	3	56.1	0.95	0.4379
2FI vs linear	75	3	25	0.38	0.7721
Quadratic vs 2FI	841.34	3	280.45	117.30	<0.0001
Residual	15.71	6	2.62		
Lack of fit tests					
Linear	940.25	11	85.48		
2FI	865.25	8	108.16		
Quadratic	23.91	5	4.78		
Pure error	0	5	0		
Model summary statistics					
Source	Standard deviation	R-squared	Adjusted R-squared	Predicted R-squared	PRESS
Linear	7.6659	0.1518	-0.0072	-0.5007	1663.5539
2FI	8.1583	0.2195	-0.1408	-4.3657	5948.1860
Quadratic	1.5463	0.9784	0.9590	0.8764	137.0434

*Significant values, df: Degree of freedom, PRESS: Sum of squares of prediction error, 2 factors interaction: 2FI

The fit summary results are presented in Table 11, contributed to find an adequate type of response surface model. The aim of sequential model sum of squares is to select the highest order polynomial where terms are significant; quadratic model type was selected to be the proper model that fit the FCCD of bioactive metabolites production by *Streptomyces lienomycini* NEAE-31, where fit summary results showed that, the quadratic model is a highly significant model with a very low probability value [(p_{model}>F)<0.0001]. The model summary statistics focus on the models that have lower standard deviation and higher adjusted and predicted R-squared; the model summary statistics of the quadratic model showed the smallest standard deviation of 1.5463 and the largest adjusted and predicted R-squared of 0.9590 and 0.8764, respectively.

Model adequacy checking: Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. The normal probability plot is important a diagnostic tool that indicates whether the residuals follow a normal distribution, in which case the points will follow a straight line expect some scatter even with normal data. Figure 7a showed that, the normality assumption was satisfied as the residuals from the fitted model were normally distributed a long a straight line for bioactive metabolites production, this indicates that the model had been validated. Also, predicted versus actual bioactive metabolites production plot as a visual diagnostic plot indicated that, there is a close agreement between the experimental results and theoretical values predicted by the model equation as shown in Fig. 7b, which confirms the adequacy of the model. As observed from Fig. 7c, the blue line indicates the current transformation (Lambda = 1) and the green line indicates the best lambda value (= 0.08), while the red lines indicate the minimum and maximum 95% confidence interval values (-0.76 and 1.05, respectively). The model is in the optimal zone since the blue line falls within the red lines. So that the model is well

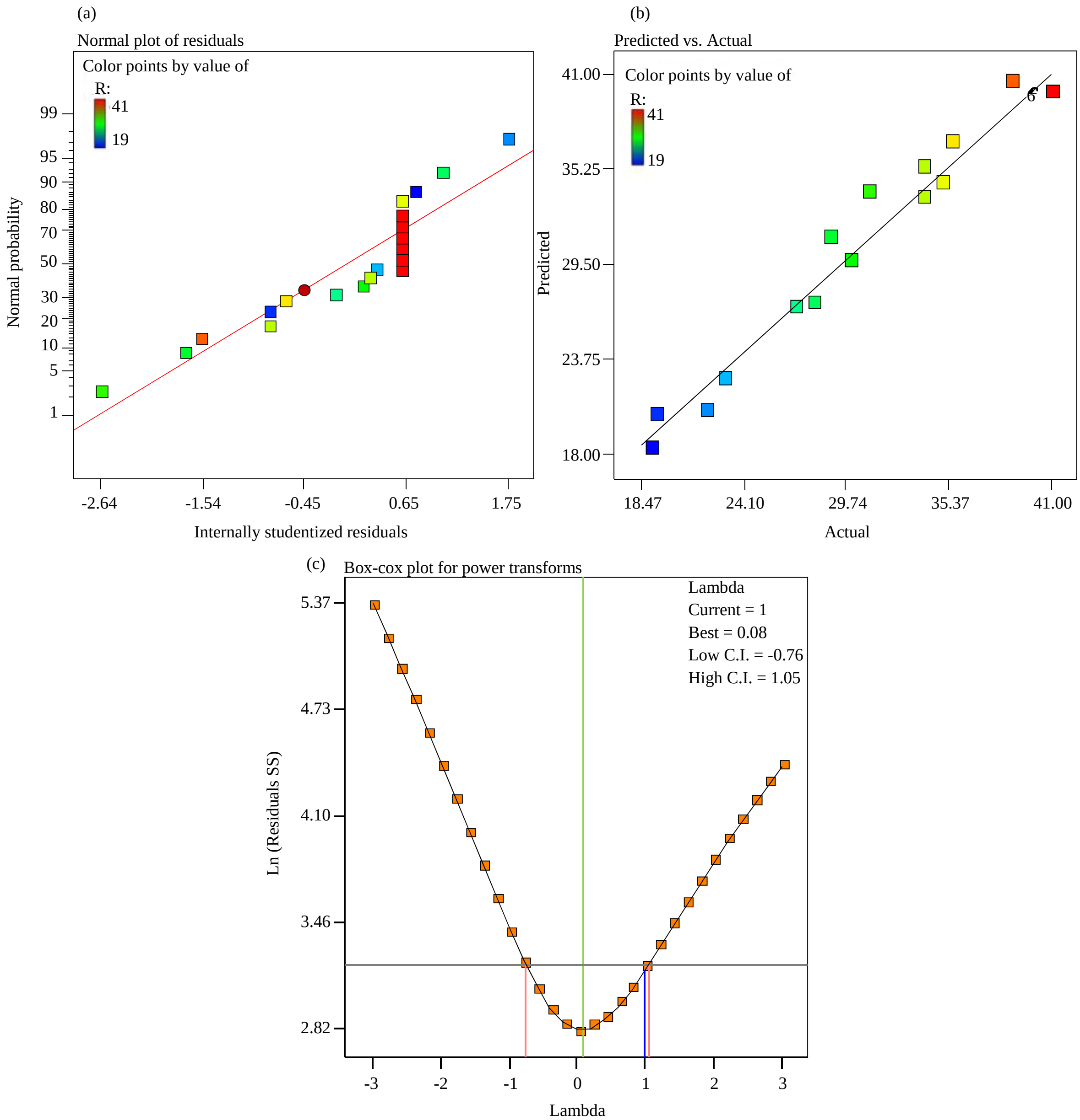

Fig. 7(a-c): (a) The normal probability plot of the residuals, (b) Correlation between the experimented values for bioactive metabolites production and predicted values determined by the second-order polynomial equation and (c) Box-Cox plot of model transformations

fit to the experimental data obtained and well satisfies the assumptions of the analysis of variance.

Three dimensional plots: The interaction effects and optimal levels of the variables were determined by plotting the three-dimensional response surface (Fig. 8a-c) when one of the variables is fixed at optimum value and the other two are allowed to vary. Figure 8a represents the three dimensional plot as function of yeast extract (X_1), $CaCO_3$ (X_2) on the production of antimicrobial metabolites. Maximum antimicrobial activity was obtained at 0.3 g L^{-1} yeast extract and 3 g L^{-1} $CaCO_3$. Further increase or decrease led to the decrease in the production of antimicrobial metabolites.

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. The nitrogen source supplied to an organism has a marked influence on the quantitative nature of the antibiotic

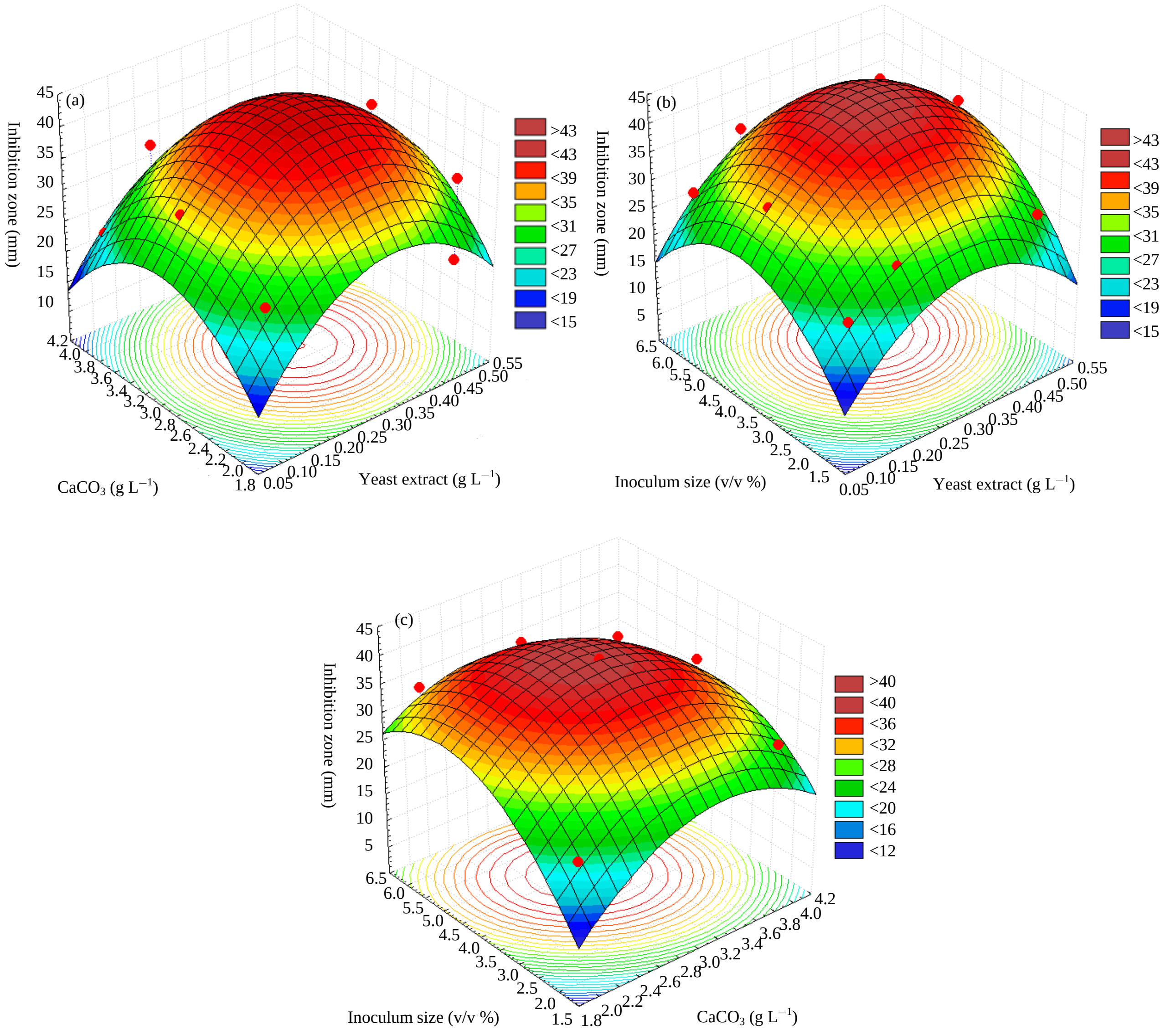

Fig. 8(a-c): Three-dimensional response surface plot for bioactive metabolites production showing the interactive effects of yeast extract (X_1), $CaCO_3$ (X_2) and inoculum size (X_3)

produced[26]. In addition, there is experimental evidence for repression of antibiotic production exerted by some nitrogen sources especially ammonium[27]. The optimal level of the yeast extract for antimicrobial agent production by *Streptomyces psammoticus* strain[28] M19 was 0.2 g L^{-1}. Our results are in accordance with Himabindu and Jetty[29] who observed that high levels of antibiotic production were in medium containing yeast extract as sole nitrogen source. In general, yeast extract is a complex nitrogen source which contains amino nitrogen (amino acids and peptides), water soluble vitamins and carbohydrates. Moreover the stimulatory effect of yeast extract on the production of natamycin may be due to the presence of trace elements in yeast extract. Kawaguchi *et al.*[30] reported that the B factor isolated from yeast extract was act as stimulatory agent for rifamycins production. On contrast, El-Naggar *et al.*[31] used starch nitrate medium containing 2 g L^{-1} potassium nitrate for the production of meroparamycin antibiotic by *Streptomyces* MAR01. Osman *et al.*[32] showed that antimicrobial productivity by *Streptomyces plicatus* was greatly affected by the used nitrogen source and the highest productivity was in the case of KNO_3.

Calcium carbonate was frequently added to the medium to counteract excess acidity and to enhance the tetracycline production but abundance might interference in the extraction procedure. About 8% $CaCO_3$, regulated the substrate pH and stimulated tetracycline production during the fermentation[33]. Calcium carbonate ($CaCO_3$) as inorganic salt favored 0.5% higher antibiotic yield[34] than the control at 1% w/w.

Figure 8b depicts the yeast extract (X_1) and inoculum size (X_3) interactions. The plot reveals that lower and higher levels of the yeast extract and inoculum size support relatively low levels of antimicrobial metabolites production. On the other hand, the maximum antimicrobial metabolites production clearly situated close to the central point of the yeast extract and inoculum size. 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[35,36]. Low inoculum may require longer time for microbial multiplication and substrate utilization to produce desired product. 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.*[37]. These results are in accordance with Ramachandran *et al.*[37] who observed 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[38]. 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[39,40]. 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[41]. On the other hand, Abdel-Fatah[42] 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.*[43] reported that maximum antibiotic production by *Streptomyces violatus* was obtained using inoculum size of 3 mL spore suspension per 50 mL 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[44].

Figure 8c represents the three dimensional plot as function of $CaCO_3$ (X_2) and inoculum size (X_3) on the production of antimicrobial metabolites. At moderate levels of $CaCO_3$ and inoculum size, the bioactive metabolites production was high. The graph pointed a decline in production level when the interaction was carried beyond high and low levels of $CaCO_3$ and inoculum size.

Model verification: In order to determine the accuracy of the model and to verify the result, an experiment under the new conditions which obtained from face-centered central composite design was preformed. The predicted optimal levels of the process variables for bioactive metabolites production by *Streptomyces lienomycini* NEAE-31 were yeast extract (0.2 g L^{-1}), $CaCO_3$ (3 g L^{-1}), inoculum size (4% v/v). The activity (41 mm) obtained from the experiment was very close to the response (40 mm) predicted by the regression model, which proved the validity of the model. The verification revealed a high degree of accuracy of the model of 97.56%, indicating the model validation under the tested conditions.

El-Naggar *et al.*[45] used the Plackett-Burman design to evaluate the effect of different culture conditions on bioactive metabolites production by a newly isolated *Streptomyces psammoticus* strain M19. Of the 15 variables examined, agitation speed, yeast extract, NaCl and KNO_3. The levels of the four medium components were further optimized using central composite design. The optimal levels for agitation speed, yeast extract, NaCl and KNO_3 were determined as 125 rpm, 0.2, 0.5 and 1.5 g L^{-1}, respectively. The antagonistic activity produced from the optimized culture conditions against multidrug-resistant *Staphylococcus epidermidis* showed about 1.37 fold increase than that obtained from the un-optimized medium. Mohamedin *et al.*[28] used two levels Plackett-Burman design for initial screening of 15 different factors for their significances on bioactive metabolites production by *Nocardiopsis chromatogenes* strain SH89. Among the variables screened, KNO_3, medium volume and agitation speed had significant effects on bioactive metabolites production. The levels of these significant variables and their interaction effects were optimized by Box-Behnken statistical design. An overall one-fold increase in the production of antimicrobial metabolites was achieved after optimization compared with that obtained in the un-optimized liquid medium. The critical control factors were selected from Plackett-Burman factorial design and the bioprocess medium was optimized by Central Composite Design (CCD) for the production of pyrrolidone antimicrobial agent from *Streptomyces* sp. MAPS15. Based on Plackett-Burman experimental design the most significant variables, such as paddy straw, $(NH_4)_2SO_4$, NaCl and pH were depicts positive effect on biomass and antimicrobial compound production[46]. Plackett-Burman design and Box and Wilson design were applied to provide an efficient approach for optimization. Statistical analysis using PBD demonstrated that NaCl, KNO_3 and K_2HPO_4 had significant positive influences on the production. In optimized medium, antimicrobial compound production was increased by 1.5 fold as compared to the basal production medium the optimal values of the variables for MRSA NaCl = 2.5, KNO_3 = 2.3 and K_2HPO_4 = 1.65 (g L^{-1})[47]. Fifteen factors were examined for their significances on production of antimicrobial metabolites using Plackett-Burman design. Among the variables screened, agitation speed (rpm), 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[48] (60 h). In addition, the optimal levels of the process 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 about one and half-fold increase in the production of antimicrobial agent[49] by *Streptomyces* sp. NEAE-1.

CONCLUSION

The present study is an step towards evaluating various nutritional and physical variables on bioactive metabolites production by the newly isolated *Streptomyces lienomycini* strain NEAE-31 using Response Surface Methodology (RSM) and search optimal conditions to attain a higher bioactive metabolites production. The RSM is one of the most practical optimization methods. This method enables us to identify the effects of individual variables and to efficiently seek the optimum conditions for a multivariable system. With this methodology, the effect of interaction of various parameters can be understood, generally resulting in high production yields and simultaneously limiting the number of experiments. Significant improvement from 25-41 mm in the production of bioactive metabolites.

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