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Optimization of Fermentation Conditions for Production of Bioactive Metabolites Effective against *Staphylococcus epidermidis* by a newly Isolated *Nocardiopsis chromatogenes* Strain SH89 using the Response Surface Methodology

Noura El-Ahmady El-Naggar[1], Attiya Hamed Mohamedin[2], A.A. Sherief[2] and Suzan Mohamed Hussien[2]

[1]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt.
[2]Department of Botany, Faculty of Science, Mansoura University, Egypt.

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The genus *Nocardiopsis*, a widespread group in phylum Actinobacteria, has received much attention owing to its ecological diversity and ability to produce a rich array of bioactive metabolites. *Nocardiopsis* sp. strain SH89 was isolated from soil sample collected from Borg El Arab city, Alexandria, Egypt and tested for its ability to produce bioactive compounds. According to the cultural, morphological, biochemical characteristics and 16S rRNA sequencing, the isolated strain is identified as *Nocardiopsis chromatogenes* strain SH89 and its sequencing product was deposited in the GenBank database under accession number KT236083. The Plackett–Burman statistical design was used for initial screening of fifteen different factors for their significance 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 by using response surface methodology compared with that obtained in the un-optimized medium. This study points the success of statistical model in developing an optimized production medium for enhanced antibacterial compound production by *Nocardiopsis chromatogenes* strain SH89.

Keywords: *Nocardiopsis* sp., bioactive metabolites, fermentation, Plackett-Burman design, Box-Behnken design, 16S rRNA, identification.

New drugs especially antibiotics had been needed to reverse the spreading of antibiotic resistant pathogens which cause infectious diseases and causes reduction in effectiveness of drug in curing of patients[1, 2]. The genus *Nocardiopsis*, a widespread group in phylum Actinobacteria, which is Gram-positive and having a genome with a high guanine and cytosine (G+C) content. The genus *Nocardiopsis* has received much attention owing to its ecological versatility, pathogenicity, and its ability to produce a rich array of bioactive metabolites. Numerous studies have shown that *Nocardiopsis* strains are ubiquitously distributed across a diverse range of environments, such as saline or alkaline habitats, deserts, marine habitats, plant tissues, animal guts, and indoor environments[3,4]. Members of the genus also produce such bioactive metabolites as

* To whom all correspondence should be addressed.
Tel.: (002)01003738444; Fax: (002)03 4593423;
E-mail,nouraelahmady@yahoo.com

methylpendolmycin[5], apoptolidin[6], griseusin D[7], lipopeptide biosurfactants[8] , thiopeptides [9] and naphthospironone A[10] .

Actinomycete taxonomy was thought to be associated with morphological and biochemical activities, which is inadequate tools in the differentiation between different species. The use of phylogenetic and molecular techniques approaches had been of great importance to the classification methods[11,12]. Recently, the identification of the species has been derived from 16S rRNA and PCR for sequence analyses. Sequences of 16S rRNA have provided actinomycetologists with a phylogenetic tree that enables the investigation for the identification [13].

Studying the factors affecting antibiotic productivity had been an important strategy for the bioprocess development. The accumulation of metabolic products was greatly influenced by the components of medium such as carbon sources, nitrogen sources and inorganic salts [14]. The classical method for optimization involves changing one variable at a time by keeping the others at fixed levels. This method was a time consuming and does not guarantee the determination of optimal conditions[15]. Optimization of factors that affect for antibiotic production performed by Plackett and Burman[16] and response surface methodology. This design allows determination of the main effect of variables with a minimum number of experiments and evaluates the relative significance of several variables simultaneously [17]. Response surface methodology has been successfully applied for optimization of the media and culture conditions in many cultivation processes for the production of primary and secondary metabolites[18]. Box-Behnken experimental design (BBD) is one class of the experimental designs for response surface methodology developed by Box and Behnken[19], is a useful method for developing second order response surface models and requires at least three levels for each factor , coded as -1, 0, and +1[20]. In BBD, the level of one of the factors is fixed at the center level while combinations of all levels of the other factors are applied [21, 22].

The aim of the present study was to identify the potent producer isolate for bioactive metabolites effective against *Staphylococcus epidermidis* using a combination of phenotypic and genotypic characteristics and to study the effect of the different fermentation variables and their interactions on the bioactive metabolites production by *Nocardiopsis chromatogenes* strain SH89.

MATERIALS AND METHODS

Isolation and culture conditions of actinomycetes

This was conducted by the soil dilution–plate technique [23]. The serial dilutions of the soil samples collected from Borg El Arab, Alexandria, Egypt were made within sterile water. The serially diluted samples were spread over the starch nitrate agar medium[24]. The agar plates were incubated at 30ºC for 7 days and the different colonies were picked up and pure culture was maintained on slopes containing starch-nitrate agar medium or stored in 20% glycerol at -20ºC.

Screening of actinomycetes for antimicrobial activities

The antimicrobial activities were tested against 7 organisms namely *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis, Shigella dysenteriae* and *Candida albicans.* These strains were kindly provided by the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Preparation of inoculum

250 mL Erlenmeyer flasks containing 50 mL of starch nitrate broth were inoculated with three disks of 9 mm diameter 7-days old *Nocardiopsis* culture grown on starch nitrate agar medium. The flasks were incubated for 7days in a rotary shaker at 30°C and 150-200 rpm[25].

Fermentation conditions

The antibiotic production by *Nocardiopsis* sp. SH89 was conducted in 250 mL Erlenmeyer flask with 50 mL of the production medium (g/L, 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 distilled water up to 1L). The production medium had adjusted to pH of 6.5 using 1.0 M HCl and sterilized at 121ºC for 20 min. It had been inoculated with 2% (v/v) of the inoculum. The flasks were incubated in a shaker at 150 rpm at 30ºC for the fermentation period of 7 days. Under aseptic conditions, the cells were separated from the

medium by centrifugation at 5000 rpm for 15 min. The supernatant was used for the analysis of antimicrobial activity.

Characterization of the *Nocardiopsis* isolates

The selected isolate was characterized using cultural, morphological and physiological methods [26]. Further characterization was done with 16S rRNA analysis.

Morphological characterization

The morphology of the spore chain and the spore surface ornamentation of the *Nocardiopsis* sp. SH89 were examined on inorganic salt/starch agar (ISP medium 4) after 14 days at 30°C. The gold-coated dehydrated specimen of actinomycete spores were examined at different magnifications with scanning electron microscope (SEM, Joel ISM 5300 at Electron Microscope Unit, Faculty of Agriculture, Mansoura University) [27].

Cultural characteristics

Aerial spore-mass color, substrate mycelia pigmentation and the production of diffusible pigments had been 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) and tyrosine agar (ISP medium 7) as described by Shirling and Gottlieb. [26] All plates were incubated at 30°C for 14 days.

Physiological and biochemical properties

The ability of isolate to utilize different carbon sources like L-inositol, D-xylose, D-fructose, D-galactose, D-mannitol, maltose, lactose, and sucrose was tested on plates containing ISP basal medium 9 [26] supplemented with a final concentration of 1% of the tested carbon sources. The plates were incubated at 30°C and results were recorded after 14 days. Melanoid pigment production was examined on peptone-yeast extract-iron agar (ISP medium 6), tyrosine agar (ISP medium 7), and tryptone-yeast extract broth (ISP medium 1) [28].

Liquefaction of gelatin was evaluated according to Gordon and Smith[29] using gelatin–peptone medium and the capacity to decompose cellulose was tested on Hutchinson liquid medium containing stripes of Whatman filter paper No.1 following the method of Crawford *et al*[30]. The ability of strain to produce α-amylase (amylolytic activity) was determined by streaking the spore suspension of the isolate onto the surface of starch–nitrate agar medium plates and incubated at 30°C for 7 days. After incubation, the plate is flooded with Gram's iodine solution and zone of clearance was observed [31]. The ability of actinomycete isolate to grow at different concentrations of NaCl was tested using starch – nitrate agar medium supplemented with 1-12% NaCl [32]. The ability to coagulate or to peptonize milk had determined as described by Kokare *et al* [31]. The ability of the isolate to inhibit the growth of seven bacterial strains, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis, Bacillus subtilis*, *Shigella dysenteriae* and *Candida albicans* strain was determined.

16S rRNA sequencing

The preparation of genomic DNA of the strain was conducted in accordance with the methods described by Sambrook *et al* [33]. 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 rRNA primer 27f (5'-AGAGTTTGATCMTGCCTCAG-3') and reverse 16s rRNA primer 1492r (5'-TACGGYTACCTT GTTACGACTT-3') and water was added up to 100 µl. The PCR-apparatus was programmed as follows, 5 min denaturation at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by a 10 min final extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product was sequenced by using two primers, 518F, 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R, 5'-TAC CAG GGT ATC TAA TCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Sequence alignment and phylogenetic analysis

The partial 16S rRNA gene sequence (1313 bp) of strain SH89 was aligned with the corresponding 16S rRNA sequences of the type

Table 1. Experimental independent variables at two levels used for the production of bioactive metabolites by *Nocardiopsis* sp. SH89 using Plackett–Burman design

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

strains of representative members of the genus *Nocardiopsis* retrieved from the Genbank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih. gov/blst) [34] and the software package MEGA4 version 2.1[35] was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm [36] based on the 16S rRNA gene sequences of strain SH89 and related organisms.

Optimization of process parameters

The optimization process by statistical approach was carried out by two steps. The first step was to identify the components of medium which have a significant effect on the production of bioactive metabolites using the Plackett-Burman design. The second step was to identify their interaction and optimum concentrations using central composite design [37].

Selection of the significant variables using Plackett-Burman design

This design allows the determination of the main effects between variables with a minimum number of experiments and evaluates the relative significance of several variables simultaneously[38]. In general this design is a two factorial one, which identifies the critical parameters required for elevated antibiotic production by screening *n* variables in *n* + 1 experiments [16].

A total of 15 independent (assigned) and four unassigned variables (commonly referred as dummy variables) were screened in Plackett–Burman experimental design of 20 trials (Table 1). Dummy variables (D_1, D_2, D_3 and, D_4) are used to estimate experimental errors in data analysis. Each variable is represented at two levels, high and low denoted by (+) and (e), respectively. The variables chosen for the present study were starch, KNO_3, K_2HPO_4, yeast extract, $CaCO_3$, $MgSO_4$, NaCl, $FeSO_4$, inoculum size, inoculum age, fermentation time, medium volume, agitation speed, pH and temperature. It is assumed that the factors have no interactions and a first-order multiple regression models are appropriate,

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

Where Y is the predicted response, $^2_0$ is the intercept term, ^2_i is regression coefficient and X_i is the coded variables.

Response surface methodology

The interaction effects between various significant variables which exerted an effect on bioactive metabolites production were optimized by Box- Benhken design[19]. In this study, the experimental design consisted of 15 trials and the independent variables were studied at five different levels, -1, 0 and +1. All the experiments were done in duplicate and the average of antimicrobial activities obtained were taken as the dependent response (Y). Predicted activity was calculated using the following second order polynomial equation,

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

Where Y was the predicted response, β_0 is the model constant, β_i is linear coefficients, β_{ii} is the quadratic coefficients and β_{ij} is the interaction coefficients and X_i is the coded levels of independent variables.

Statistical analysis

Minitab14 had used for experimental designs. The experimental data obtained had subjected to multiple linear regressions using Microsoft Excel 2007 to evaluate the analysis of variance (ANOVA) and to estimate main effects, *t*-value, *p*- value and the confidence levels. The

quality of the model was expressed via the correlation coefficient (R) and the adjusted R^2. 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 DISCUSSIONS

Antimicrobial activity

The antimicrobial activity of the secondary metabolites produced by *Nocardiopsis* sp. SH89 was tested against a number of Gram-positive and Gram-negative bacteria in addition to *Candida albicans* (Table 2). There was a strong activity against *Staphylococcus epidermidis*. The diameter of inhibition zones obtained with *Staphylococcus epidermidis*, *Staphylococcus aureus, Bacillus subtilis, E. coli* and *C. albicans* were 30, 10, 30, 15 and 17 mm respectively. There was no activity against *Pseudomonas aeruginosa* and *Shigella dysenteriae*.

Characterization of the selected isolate

The *Nocardiopsis* sp. SH89 was characterized by morphological, cultural, biochemical methods and 16S rRNA analysis.

Cultural Characteristics

The cultural characteristics of the isolate are represented in Table 3. *Nocardiopsis* sp. SH89 exhibited moderate growth on tryptone-yeast extract agar (ISP-1), yeast extract-malt extract-agar (ISP-2) and inorganic salt starch agar (ISP-4). The growth was well on starch nitrate agar medium, glycerol asparagine agar medium (ISP-5), peptone-yeast extract iron agar medium (ISP-6) and tyrosine agar medium (ISP-7), while it was poor on nutrient agar medium. White aerial mycelium was found on tryptone yeast extract agar medium, glycerol asparagine agar medium and peptone yeast extract

Table 2. The antimicrobial activities of the bioactive metabolites produced by *Nocardiopsis* sp. SH89

Microorganisms	Diameter of inhibition zone (mm)
Gram- positive bacteria	
Staphylococcus epidermidis	30
Staphylococcus aureus	10
Bacillus subtilis	30
Gram-negative bacteria	
Shigella dysenteriae	----
Pseudomonas aeruginosa	---
Escherichia coli	15
Yeast	
Candida albicans	17

Table 3. Cultural characteristics of the selected *Nocardiopsis* sp. SH89

Medium	Growth	Aerial mycelium	Color of Substrate mycelium	Diffusible pigment
Starch-nitrate agar	Well	Brownish white	Brown	Brown
Tryptone yeast extract agar (ISP medium 1)	Moderate	White	Brown	Brown
Yeast extract malt agar (ISP medium 2)	Moderate	Pink	Brown	Brown
Inorganic salt starch agar (ISP medium 4)	Moderate	Brownish white	Brown	Brown
Glycerol-asparagine agar (ISP medium 5)	Well	White	White	Non pigmented
Peptone-yeast extract iron agar(ISP medium 6)	Well	White	Yellowish brown	Brown
Tyrosine agar medium (ISP medium 7)	Well	Brownish white	Brown	Brown
Nutrient agar medium	Poor	Not distinctive	Not distinctive	Non pigmented

iron agar medium whereas the aerial mycelium was brownish white on starch nitrate agar medium, inorganic salt starch agar medium and tyrosine agar medium and pink on yeast extract-malt extract-agar. No aerial mycelium had been formed on nutrient agar medium. The substrate mycelium ranges from white to brown, not distinctive or yellowish brown on different tested media. A brown pigment is found in starch nitrate agar medium, tryptone-yeast extract agar medium, yeast extract-malt extract-agar medium, inorganic salt starch agar medium, peptone-yeast extract iron agar medium and tyrosine agar medium. Sivakumar[39] reported that the cultural properties had been used as markers by which an individual strain can be recognized.

Morphological characteristics

The strain exhibited typical morphological characteristics of the genus *Nocardiopsis*. Morphological observation of the isolate showed that aerial and vegetative mycelia were abundant, well developed. Spores with smooth surface (Fig.1). The microscopic examination of the selected isolate revealed that spore chain was morphologically related to section rectiflexibiles.

Biochemical characterization

The details of biochemical and physiological properties of the isolate are given in Table 4. The isolate exhibited positive response to starch hydrolysis, casein hydrolysis, gelatin liquefaction, melanin production but negative response to cellulose decomposition. The Utilization of carbon sources could be used as a tool for species determination[40]. The isolate was able to utilize the carbon sources D-glucose, D-xylose, D-mannitol, D- galactose and sucrose but can't utilize L-arabinose, dextrose, ribose, lactose, L-inositol and D-raffinose. Optimum growth of the isolate occurred at the pH 6.5. The isolate exhibited sodium chloride tolerance up to 4 %, accordingly, the isolate could be placed in the intermediate salt tolerance group according to Tresner *et al*[32].

16S rRNA gene sequence comparisons and phylogenetic analysis

The 16S rRNA gene sequence (1313 bp) was determined for strain SH89. A BLAST search[34] of the Genbank database using this sequence showed its similarity to that of many species of the genus *Nocardiopsis*. A phylogenetic tree (Fig. 2) based on 16S rRNA gene sequences of members of the genus *Nocardiopsis* was constructed according to the bootstrap test of neighbor-joining algorithm method of Saitou and Nei [36] with MEGA4[35]. This tree shows the close phylogenetic association of strain SH89 with certain other *Nocardiopsis* species. Phylogenetic analysis indicated that the strain SH89 consistently falls into a clade together with *Nocardiopsis compostus* strain, KS8 (GenBank/EMBL/ DDBJ accession No. AF360733.1), *Nocardiopsis chromatogenes* strain YIM90109 (GenBank/EMBL/ DDBJ accession No. NR_043032.1), *Nocardiopsis gilva* strain YIM90087 (GenBank/EMBL/ DDBJ accession No. NR_043029.1), *Nocardiopsis rosea* YIM90094 (GenBank/EMBL/ DDBJ accession No. NR_043030.1) and *Nocardiopsis trehalosi* strain VKM Ac_942 (GenBank/EMBL/ DDBJ accession No.NR_024958.1).

On the basis of the collected data and in view of the comparative study of the recorded properties of isolate SH89 in relation to the closest related species of the genus *Nocardiopsis* it is most closely related to the type strains of

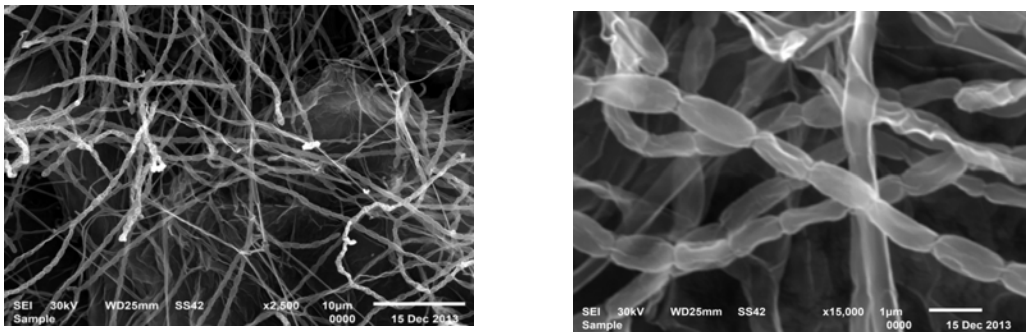

Fig. 1. Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of the strain grown on inorganic salt-starch agar medium for 14 days at 30 °C a magnification of 2500, 15000 X

Nocardiopsis chromatogenes [41]. Comparison of the morphological, cultural and physiological characteristics of strain SH89 and its closest phylogenetic neighbours *Nocardiopsis chromatogenes* revealed significant similarities in that it produced spore chains in section rectiflexibiles, spore surface is smooth, spores are elongated in shape. Aerial mass color belongs to the white color series on most media. Melanoid pigments are formed in peptone-yeast-iron agar, tyrosine agar and tryptone-yeast broth. The isolate was able to utilize the carbon sources D-glucose, D-xylose, D-mannitol, D- galactose and sucrose but can't utilize dextrose. As similar to *Nocardiopsis chromatogenes*, the isolate exhibited positive response to starch hydrolysis, gelatin liquefaction and melanin production but cellulose decomposition was negative. Therefore, this strain was identified as *Nocardiopsis chromatogenes* strain SH89 and its sequencing product was deposited in the GenBank database under accession number KT236083.

Table 4. Summary of the cultural, morphological and physiological properties of the experimental actinomycete isolate

Characteristic	Actinomycete isolate SH89	*Nocardiopsis chromatogenes**
Aerial mycelium on most media	White	White
Substrate mycelium on most media	Brown	Light reddish brown to deep reddish brown
Spore chain morphology	Long spore chains, suggestive to section rectiflexibiles	Long spore chains
Spore shape	Spores are elongated with smooth surface and nonmotile	Spores are smooth- surfaced and non motile
Melanin production	+	+
NaCl tolerance (w/v)	4%	0-18%, optimum growth is at 5-8%
Growth on sole carbon sources (1%, w/v),		
D-glucose	+	+
D- xylose	+	+
L- arabinose	–	+
Dextrose	–	–
Ribose	–	+
D- mannitol	+	+
D-galactose	+	+
Lactose	–	+
L- inositol	–	+
D- raffinose	–	+
Sucrose	+	+
Enzyme activities,		
α–amylase(starch hydrolysis)	+	+
Cellulase (cellulose decomposition)	–	–
Casienase (casein hydrolysis)	+	
Gelatinase (gelatin Liquefaction)	+	–
Antimicrobial activities against,		
P. aeruginosa and Shigella dysenteriae	–	
B. subtilis, S. epidermidis, C. albicans, S. aureus , E. coli and C. albicans	+	

*Data for reference species were taken from Bergey's Manual® of Systematic Bacteriology-volume five the actinobacteria (Goodfellow *et al.*[41]).
Abbreviations, +, Positive, –, Negative, ±, Doubtful, Blank cells, no data available.

Screening of parameters using Plackett- Burman design

The experiment had been conducted in twenty runs to study the effect of the selected factors on the production of bioactive metabolites by *Nocardiopsis chromatogenes* strain SH89. The design matrix selected for the screening of significant variables for bioactive metabolites production and the corresponding responses are shown in Table 5. All the trails were performed in duplicate and the average were taken as response. The maximum bioactive metabolites activity (37 mm) was achieved in the run number 7, while the minimum bioactive metabolites activity (8 mm) was observed in the run number 20. The statistical analysis of Plackett-Burman design was performed (Table 6). The determination coefficient (R^2) provides a measure of how much variability of the observed response can be explained by the experimental factors and their interactions. R^2 values will always be a number between 0 and 1, with values close to 1 indicating a good degree of fit. In this design the value of $R^2 = 0.9884$ indicates that 98.84% of the variability of response was due to the given variables and only 1.16% of the variation had not explained by the independent variables.

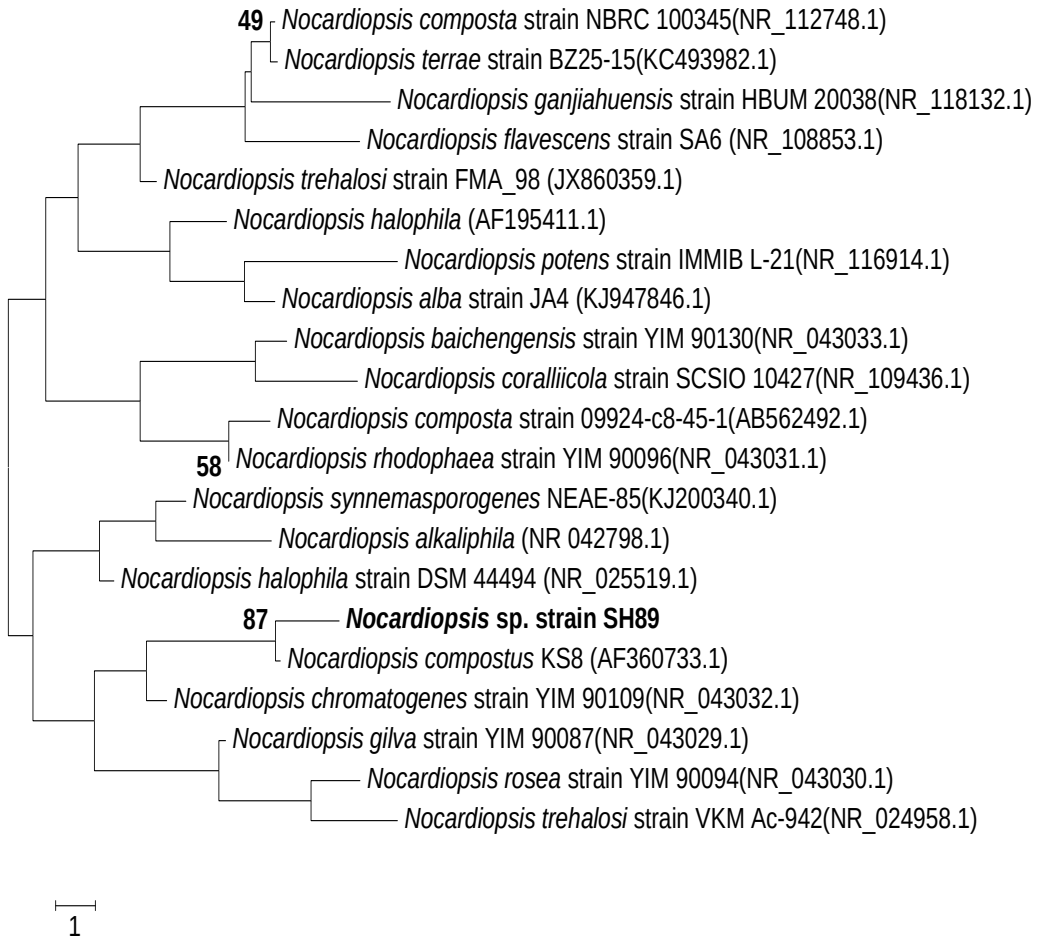

Fig. 2. The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm based on the 16S rRNA gene sequences of strain SH89 and related species of the genus *Nocardiopsis.* Only bootstrap values above 50 %, expressed as percentages of 1000 replications, are shown at the branch points. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA4. Bar, 5 substitution per nucleotide position

In addition, the adjusted R^2 =0.9451 had very high value which indicate a high significance of the model. The correlation coefficient (R = 0.9942) indicates excellent correlation between the variables which indicate a good relation between experimental and predicted values (Table 6). With the respect to the main effect of each variable (Fig. 3) nine variables namely starch, KNO_3, NaCl, $MgSO_4$, pH, agitation speed, medium volume, inoculum size and fermentation time positively affected the bioactive metabolites production, while other parameters negatively affect the production. In the response surface methodology the variables which had a positive effect on bioactive metabolites production were fixed at high levels, while others that had a negative effect were fixed at low levels. The Pareto chart illustrates the order of significance of the variables affecting bioactive metabolites production in Plackett-Burman Experimental Design (Fig. 4). Among the 15 variables, KNO_3 and medium volume showed the highest positive significance by 18.47%. Next to both, agitation speed showed positive effect by 12.8 %. Temperature showed the highest negative effect by 10 % and $CaCO_3$ showed negative effect by 5.2%.

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 (*F* model = 22.839) and a very low probability value (*P* model > *F* = 0.0040). The *t* and *p* values (Table 6) illustrate the significance of each variable. The larger magnitude of *t*-value and the smaller *p*-value indicate significant effect of a given variable[42]. In this experiment, the variables that had *p*-value less than or equal 0.05 have significant effect on the response. On the basis of the calculated *t*-values, KNO_3, medium volume and agitation speed were the most significant with a positive effect while temperature and $CaCO_3$ had a negative effect. KNO_3, medium volume and agitation speed were used for further optimization by central composite design.

By neglecting the non-significant variables which were insignificant, the first order polynomial equation was,

$$Y= 22.5+4.6\,(X_2) +2.7\,(X_5) -1.3\,(X_7) - 2.5\,(X_{10}) +3.2(X_{11}) +4.6(X_{12}) \quad ...(3)$$

Where Y is the response (bioactive metabolites production), X_2, X_5, X_7, X_9, X_{10}, X_{11}

Table 5. Twenty-trial Plackett–Burman experimental design for evaluation of fifteen independent variables with coded values along with the observed bioactive metabolites activity.

Trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	D_1	D_2	D_3	D_4	Inhibition zone (mm)
1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	28
2	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	24
3	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	10
4	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	24
5	1	1	1	1	-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	1	-1	-1	1	33
7	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	37
8	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	17
9	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	26
10	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	30
11	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	17
12	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	9
13	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	27
14	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	25
15	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	20
16	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	36
17	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	28
18	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	9
19	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	23
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8

X_1–X_{15} represent independent (assigned) variables, D_1–D_4 are the dummy variables (unassigned), '1' is for high level of variables and '-1' is for low level of variables

and X_{12} are KNO_3, NaCl, $CaCO_3$, temperature, agitation speed and medium volume respectively. It can be seen that KNO_3, NaCl, agitation speed and medium volume exhibit positive effect on bioactive metabolites production, while $CaCO_3$ and temperature maintained a negative effect.

It had been necessary to check the model to ensure that it provides an adequate approximation to the real system (Fig 5A-C). The normal probability plot (Fig 5A) of the residuals shows that the points close to the line, so the residuals appear to be normally distributed which indicate that the model was fitted to the experimental results. Fig. 5B which presents a plot of predicted vs observed values of response, showed a satisfactory correlation between the experimental values and predicted values where in, the points gathered around the diagonal line indicates the good fit of the model. The residual plot (Fig. 5C) 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.

Optimization by Box-Behnken design

The Box-Behnken design (BBD) was employed to study the optimal levels and interactions between the significant variables. Other variables that not selected in Box-Behnken design were maintained at constant level which gave maximum production in Plackett-Burman design. A total of 15 experiments with different combinations of KNO_3, medium volume and agitation speed were evaluated in Table 7. Close similarities is evident between the measured inhibition zones and the predicted ones (Table 7).

Multiple regression and analysis of variance (ANOVA)

Multiple regression analysis had been used to analyze the data (Table 8). The goodness

Table 6. Statistical analysis of Plackett-Burman design showing coefficients, *t*-test, and *p*-values for each variable affecting bioactive metabolites production and analysis of variance (ANOVA)

Variables	Coefficients	Main effect	*t* -value	*p*-value	Confidence Level
Intercept	22.5	45	48.809	0.000	100.00
Starch (g/L)	0.9	1.8	1.952	0.123	87.74
KNO_3 (g/L)	4.6	9.2	9.979	0.001	99.94
K_2HPO_4 (g/L)	-0.4	-0.8	-0.868	0.435	56.55
Yeast extract(g/L)	-0.3	-0.6	-0.651	0.551	44.93
NaCl (g/L)	2.7	5.4	5.857	0.004	99.58
$MgSO_4.7H_2O$(g/L)	0.8	1.6	1.735	0.158	84.23
$CaCO_3$ (g/L)	-1.3	-2.6	-2.820	0.048	95.22
FeSO4 (g/L)	-0.9	-1.8	-1.952	0.123	87.74
pH	1.1	2.2	2.386	0.075	92.45
Temperature (°C)	-2.5	-5	-5.423	0.006	99.44
Agitation speed (rpm)	3.2	6.4	6.942	0.002	99.77
Medium volume (mL/250 mL flask)	4.6	9.2	9.979	0.001	99.94
Inoculation size (%)	0.2	0.4	0.434	0.687	31.32
Inoculation age (h)	-0.5	-1	-1.085	0.339	66.09
Fermentation time (d)	1	2	2.169	0.096	90.41

Analysis of variance (ANOVA)

	df	*SS*	*MS*	*F*	Significance *F*
Regression	15	1456	97.06667	22.83922	0.004038
Residual	4	17	4.25		
Total	19	1473			

t – student's test, *p* – corresponding level of significance

Multiple R 0.9942, R Square 0.9884, Adjusted R Square 0.94518, *df* - Degree of freedom, *SS*- Sum of squares, *MS*- Mean sum of squares, *F* – Fishers's function, Significance *F* – Corresponding level of significance.

Table 7. Box–Behnken factorial experimental design, representing the response of antimicrobial activity as influenced the selected variables and the levels of these variables

Trials	KNO_3	Agitation speed	Medium volume	Antimicrobial activity Experimental	Predicted
1	-1	-1	0	12	11.1
2	1	-1	0	27	28.1
3	-1	1	0	15	14.8
4	1	1	0	15	16.8
5	-1	0	-1	27	28.0
6	1	0	-1	27	25.9
7	-1	0	1	14	14.1
8	1	0	1	29	27.1
9	0	-1	-1	27	26.9
10	0	1	-1	25	24.2
11	0	-1	1	25	25.8
12	0	1	1	24	24.1
13	0	0	0	40	40.0
14	0	0	0	40	40.0
15	0	0	0	40	40.0

Variables level	g/L	rpm	mL/250 mL conical flask
-1	1	150	50
0	2	175	75
1	3	200	100

Table 8. Statistical analysis of Box–Behnken design showing coefficients, t-test, and p-values for each variable affecting bioactive metabolites production and analysis of variance (ANOVA)

	Coefficients	*t*- value	*P*-value	Confidence Level
Intercept	39.98492	44.65738	1.06E-07	99.99
X_2	5.590452	9.755639	0.000192	99.99
X_{11}	-1.08573	-1.92081	0.112812	99.88
X_{12}	-0.32382	-0.57289	0.59152	99.40
X_2X_{11}	-2.92145	-3.58412	0.015806	99.98
X_2X_{12}	6.602357	8.099962	0.000465	99.99
$X_{11}X_{12}$	0.25	0.319152	0.762514	99.23
X_2X_2	-10.0327	-12.3363	6.2E-05	99.99
$X_{11}X_{11}$	-11.4094	-12.6549	5.47E-05	99.99
$X_{12}X_{12}$	-3.31419	-3.67598	0.014354	99.98

Analysis of variance (ANOVA)	*df*	*SS*	*MS*	*F*	Significance *F*
Regression	9	1176.128	130.6809	53.24345	0.000197
Residual	5	12.27201	2.454403		
Total	14	1188.4			

t – student's test, *p* – corresponding level of significance Multiple R 0.9948, R Square 0.98967, Adjusted R Square 0.97108; *df* - Degree of freedom, *SS*- Sum of squares, *MS*- Mean sum of squares, *F* – Fishers's function, Significance *F* – Corresponding level of significance

of the model was checked by the coefficient of determination (R^2) which was found to be 0.9896 (Table 8) this indicates that the variation of sample 98.96 % had been attributed to the selected variables and only 1.14% of the total variance couldn't be explained by the model. A regression model has R^2- value higher than 0.9 was considered to have a very high correlation. [43] Therefore, the obtained R^2 value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for antimicrobial metabolites production in the present study.

The Fisher's test (53.24345) and very low probability value (0.000197) results showed that the model had a highly significance values. The significance of each variable was demonstrated by *t*-values and *p*-values which are listed in Table 8. The *p*–values denotes the significance of the coefficients and also important in understanding the pattern of mutual interaction between the variables. It can be seen from the degree of significance that the linear coefficients of X_2 and the quadratic effect of three independent variables had a significance effect on the production. The probability values of the coefficients suggest that among the three variables studied X_2, X_{11} and X_2, X_{12} showed maximum interaction between the two variables. On the other, the linear coefficients of X_{11}, X_{12} and the interaction between X_{11}, X_{12} has non-significant effect on bioactive metabolites production.

In order to evaluate the relationship between the variables to determine the maximum antimicrobial metabolites production corresponding to the optimum levels of X_2, X_{11} and X_{12}, a second- order polynomial model was proposed to calculate the optimum level of these variables. By applying the multiple regression analysis on the experimental data, the second order polynomial equations that define the predicted response (Y) in terms of the independent variables (X_2, X_{11} and X_{12}) was obtained,

$$Y = 39.98 + 5.59\,X_2 - 1.08\,X_{11} - 0.32X_{12} - 2.92\,X_2X_{11} + 6.60\,X_2X_{12} + 0.25\,X_{11}X_{12} - 10.03X_2^2 - 11.40\,X_{11}^2 - 3.31\,X_{12}^2 \quad ...(4)$$

Where Y is the predicted response, X_2 the coded value of KNO_3, X_{11} the coded value of agitation speed and X_{12} the coded value of medium volume.

The interaction effects and optimal levels of the variables were determined by plotting the three-dimensional response surface (shown in Figs. 6A–C) when one of the variables is fixed at optimum value and the other two are allowed to vary. Fig. 6A represents the three dimensional and Contour plots as function of KNO_3 and agitation speed on the production of antimicrobial metabolites. Maximum antimicrobial activity of 40 mm was obtained at the 2 g/L KNO_3 and 175 rpm agitation speed. Further increase or decrease led to the decrease in the production of antimicrobial metabolites. In this respect KNO_3 was detected to be significant factor which had a positive effect on the bioactive metabolites 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

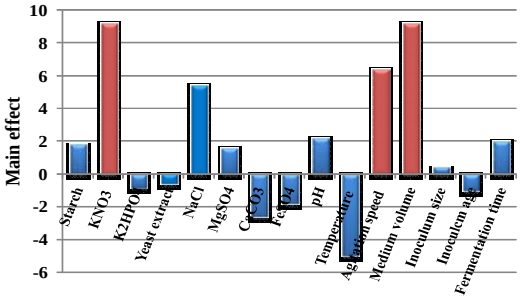

Fig. 3. The main effects of the fermentation medium constituents on bioactive metabolites production according to the Plackett-Burman experimental results (the red color represent the most significant variables)

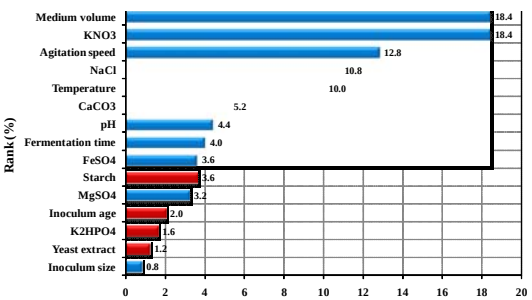

Fig. 4. Pareto chart illustrates the order of significance of the variables affecting the bioactive metabolites production, Ranks (%) values ranging from 0.80 to 18.4

experimental evidence for repression of antibiotic production exerted by some nitrogen sources and especially ammonium[44]. Osman *et al*.[45] 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. The optimal level of KNO_3 for antimicrobial agent production by *Streptomyces* sp. NEAE-1 were 3 g/L[46]. El-Naggar *et al*.[47] used starch nitrate medium containing 2 g/L potassium nitrate for the production of meroparamycin antibiotic by *Streptomyces* MAR01. High levels of antibiotic production were observed in medium containing yeast extract as sole nitrogen source[48]. 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.*[49] reported that the B factor isolated from yeast extract was act as stimulatory for rifamycins production. Generally, suitable agitation speed lead to sufficient supply of dissolved oxygen in the media[50]. Nutrient uptake by bacteria also will be increased[51]. In addition, EL-Naggar *et al.*[52] reported that the maximum antimicrobial metabolites production by *Streptomyces anulatus* NEAE-94 was obtained using high level of agitation speed (250 rpm/min).

Fig. 6B depicts the medium volume and KNO_3 interactions. The plot reveals that lower and higher levels of the medium volume and KNO_3 concentration 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 medium volume and KNO_3 concentration. The Contour plot between the medium volume and KNO_3 (Fig. 6B) indicate that there was a significant interaction with positive effect between these variable set on antimicrobial metabolites production. The maximum antimicrobial metabolites production was obtained at 2 g/L KNO_3 and 75 mL/250 mL conical flask medium volume. Medium volume had an effect on fermentation, it was obvious that high aeration was appropriate aeration adjustment should be adopted to promote the efficiency of substrate utilization efficiency and increase the production. Although a larger medium volume initially contains more oxygen, nutrients and space for growth of bacteria, the void in the container and subsequently oxygenation of the medium will be decreased.

Fig. 6C represents the three dimensional and Contour plots as function of medium volume and agitation speed on the production of

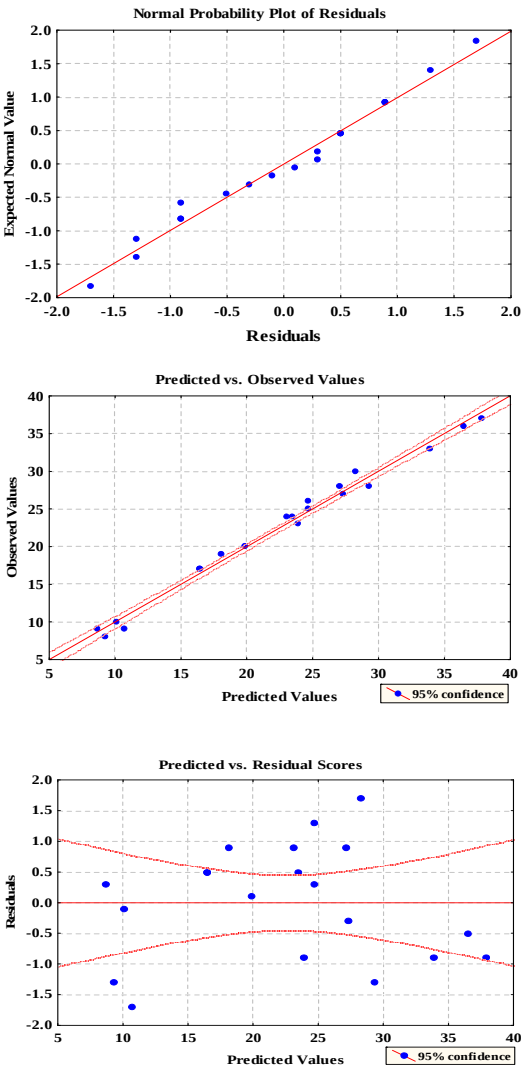

Fig. 5. A) The normal probability plot of the residuals. B) Correlation between the observed and predicted values of the bioactive metabolites activity of *Nocardiopsis* sp. SH89 determined by the first-order polynomial equation. C) Plot of residuals against predicted values for bioactive metabolites production.

beneficial to cell growth by increasing cell growth rate and production of secondary metabolites[53, 54]. This might be explained by the fact that high aeration rate intensified cell respiration and energy metabolism, which would trigger more carbon flux flow to tricarboxylic acid cycle. Therefore,

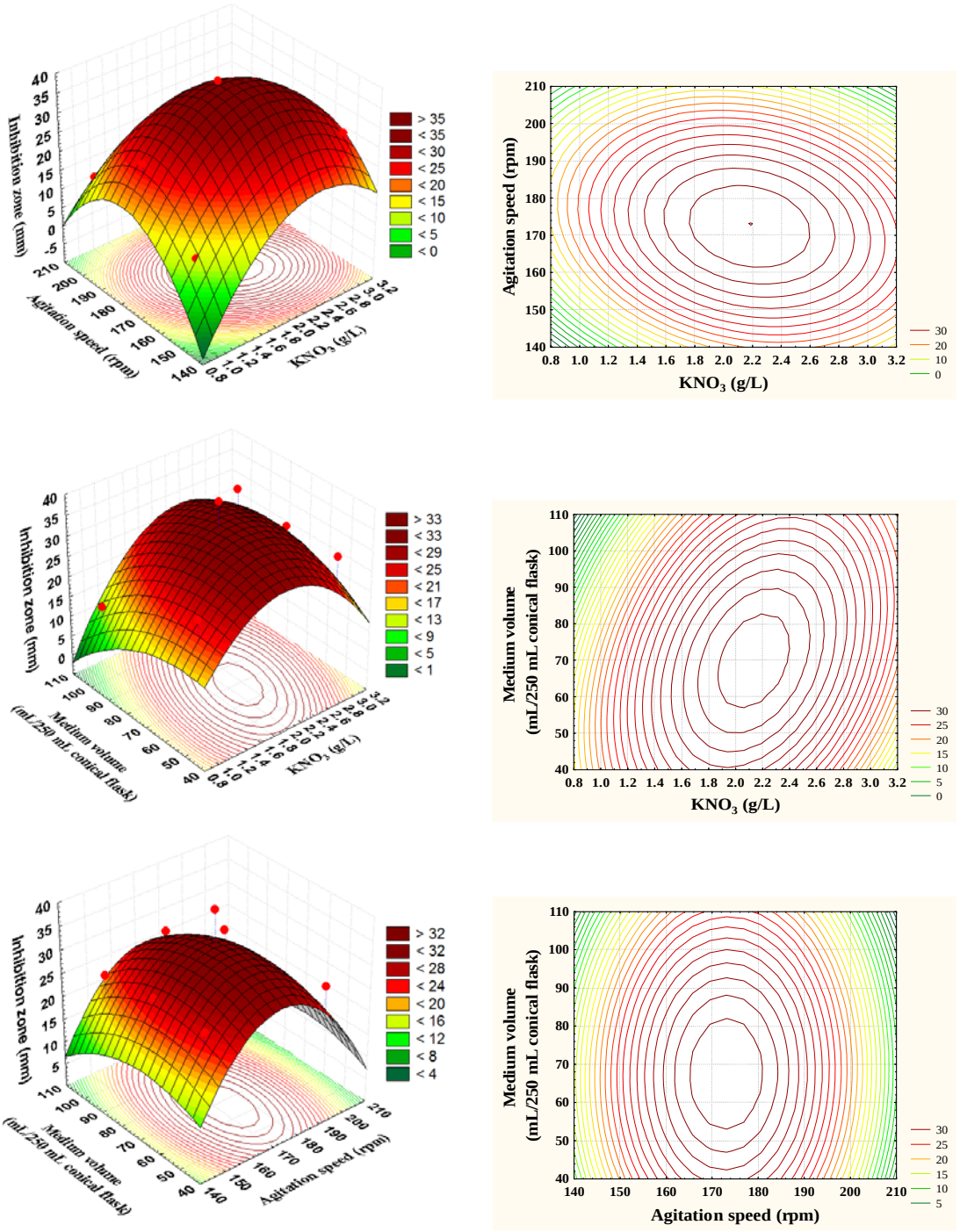

Fig. 6. Three dimensional response surface and Contour plots showing the interactive effects of independent variables agitation speed, medium volume and KNO_3 on bioactive metabolites production

antimicrobial metabolites. Higher levels of the production were attained with central point of medium volume and agitation speed. Further increase in the medium volume and agitation speed led to the decrease in the production of antimicrobial metabolites. In addition, the interaction terms between these variables were not significant, indicating that there is no significant correlation between each two variables and that they did not help much in increasing the production of antimicrobial metabolites.

Verification of the model

Optimal values of the tested variables obtained from the optimization had been verified experimentally and compared with the predicted data. The measured antimicrobial activity was 40 mm, where the predicted value from the model was 41.12 mm. The verification revealed a high degree of accuracy of the model of more than 97. 27 %, indicating the model validation under the tested conditions. The predicted optimal levels of the process variables for antimicrobial metabolites production were agitation speed (175 rpm), KNO_3 (2 g/L), medium volume (75 mL/250 mL flask).

CONCLUSION

This study had been conducted to investigate the antimicrobial potential of *Nocardiopsis* sp. isolate SH89 which isolated from Borg El Arab, Alexandria, Egypt. The cultural, morphological and physiological characteristics were studied. Factors that affect the antimicrobial activities were investigated and assessed using the statistical experimental design. The antimicrobial metabolites production had been found to be significantly influenced by agitation speed, KNO_3 and medium volume. The maximum extracellular antimicrobial metabolites production (40 mm) was achieved with the following optimized factors, starch 10 g, KNO_3 2g, K_2HPO_4 0.5g, yeast extract 0 g, NaCl 0.1g, $MgSO_4$ 0.1g, $CaCO_3$1g, $FeSO_4$ 0.01g, pH 6.5, temperature 25°C, agitation speed (180 rpm/min), medium volume 50 mL, inoculum size 2 % (v/v), inoculation age 48h, and fermentation time 5 days. Validation experiments had carried out to verify the adequancy and accuracy of the model, the results showed that the predicted value agreed well with the experimental values and about one fold increase compared to the original medium was obtained.

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