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Modeling-based optimization approaches for the development of Anti-*Agrobacterium tumefaciens* activity using *Streptomyces* sp TN71

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ABSTRACT

A new aerobic bacterium TN71 was isolated from Tunisian Saharan soil and has been selected for its antimicrobial activity against phytopathogenic bacteria. Based on cellular morphology, physiological characterization and phylogenetic analysis, this isolate has been assigned as *Streptomyces* sp. TN71 strain. In an attempt to increase its anti-*Agrobacterium tumefaciens* activity, GYM + S (glucose, yeast extract, malt extract and starch) medium was selected out of five different production media and the medium composition was optimized. Plackett–Burman design (PBD) was used to select starch, malt extract and glucose as parameters having significant effects on antibacterial activity and a Box–Behnken design was applied for further optimization. The analysis revealed that the optimum concentrations for anti-*A. tumefaciens* activity of the tested variables were 19.49 g/L for starch, 5.06 g/L for malt extract and 2.07 g/L for glucose. Several Artificial Neural Networks (ANN): the Multilayer perceptron (MLP) and the Radial basis function (RBF) were also constructed to predict anti-*A. tumefaciens* activity. The comparison between experimental with predicted outputs from ANN and Response Surface Methodology (RSM) were studied. ANN model presents an improvement of 12.36% in terms of determination coefficients of anti *A. tumefaciens* activity. To our knowledge, this is the first work reporting the statistical versus artificial intelligence based modeling for optimization of bioactive molecules against phytopathogens.

1. Introduction

Diseases caused by plant pathogenic bacteria significantly contribute to the overall loss in crop yield worldwide [1]. Despite the existence of defense mechanisms, plants are exposed to attack by plant pathogenic microorganisms. Many phytopathogenic bacteria including *A. tumefaciens* causing neoplastic diseases known as crown gall reduce the shelf life and market values of food commodities and make them unfit for human consumption [2–4]. For many years, a variety of different synthetic chemicals have been extensively used to inhibit the growth of plant pathogenic bacteria. Excessive use of chemicals was not always efficient against soil borne pathogens and led to environmental pollution, pathogen resistance, and increased risk to human and animal health [5]. In addition, strict regulations on agrochemical consumption will limit its application in the near future [6]. Therefore, new

biocontrol options against microbial pathogens are required to have more effective resistance to the pathogens and ensure sustainable crop production. Therefrom, search for novel bioactive compounds with strong activity against phytopathogens is required.

Members of the order Actinomycetales, most notably *Streptomyces* species provide a rich source of natural products that may have potential agricultural uses [6]. Several studies have demonstrated the ability of *Streptomyces* to produce a huge variety of antibacterial secondary products and their effectiveness against some plant pathogens [7]. Secondary metabolite production e.g., antibiotics, is dependent on medium composition. Hence, the optimization of the cultural medium and the cultural conditions for growth and antimicrobial activity of biocontrol agents is important for industrial applications [8]. Medium optimization for improving antimicrobial metabolite production using statistical methods allowed detection of optimal levels of factors in less

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time. For this, statistical or mathematical designs are used to reduce the number of experiments and to increase the precision of the results. Response surface methodology, a combination of mathematical and statistical techniques, was used for modeling and analysis of problems associated with multivariable systems. It can also be used to estimate the relationship between the responses (i.e., antimicrobial activity) and the experimental parameters (i.e., concentration of the medium components). The RSM has been successfully applied for optimization of medium components for metabolite production [8] and evaluate the relative significance of several variables simultaneously [8,9]. One drawback of RSM is that it is mainly restricted to quadratic nonlinear correlation, whereas biological process may show more complex nonlinear dependencies. On the contrary, ANNs successfully address and overcome the limitations of RSM. ANNs, complex mathematical models, are effective in handling data with noise, mimic biological neural networks, perform better than statistical/regression based models and accurately predict and model highly non-linear and complex biological processes [10]. MLP and RBF neural network architectures are possibly the most used ANNs [11]. MLP and the RBF neural network structures have been employed in making predictions [11]. Due to the non-linear efficiencies of these networks, they are considered good estimators providing very accurate results.

Articles on the comparison of RSM and ANN techniques for culture medium optimization simultaneously for anti-*A. tumefaciens* activity by *Streptomyces* are not available in the literature. Therefore, the present study deals with comparison between two optimization techniques: RSM and ANN that were used to enhance the antibacterial activity against *A. tumefaciens* activity by *Streptomyces* TN 71 culture medium optimization.

2. Material and methods

2.1. Identification of actinobacteria strain TN71

The actinomycete strain TN71 was isolated from Saharan soil of Tunisia and selected among a group of actinomycetes that has the capability to produce antimicrobial agents against Gram-positive and Gram-negative bacteria and fungi. The culture was grown and maintained on ISP medium 2 broth (malt extract 10 g, yeast extract 4 g, glucose 4 g, distilled water 1000 mL, pH 7.2).

The morphological and cultural characteristics were examined for 14 days-old cultures grown on various ISP (International Streptomyces Project) media: yeast extract–malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3) and inorganic salts–starch agar (ISP medium 4) [12], and also on Bennett medium [13].

For the physiological study, twenty eight tests were used including the production of melanoid pigments on ISP medium 6 and ISP medium 7, the ability of the *Streptomyces* strains to use twelve compounds as sole carbon sources and nine compounds as sole nitrogen sources for energy, the sensitivities to sodium chloride (5, 6, 7 and 10% w/v) and the growth at 45 °C [14].

For molecular analysis, DNA was prepared using the procedure recommended by Hopwood et al., in 1985 [15]. DNA isolation and manipulation, PCR amplification, DNA sequencing, and analysis of TN71 16S rRNA gene were performed as described by Elleuch et al. in 2010 [16]. Nucleotide sequence of the 16S rRNA gene of TN71 strain was determined on both strands by an automated 3100 Genetic Analyzer (Applied Biosystems) using specific primers. Homology search was performed using BLAST Search algorithm.

Phylogenetic analyses were conducted using MEGA version 7.0 [17]. The 16S rRNA gene sequence of TN71 strain was aligned using the CLUSTAL W program [18] against corresponding nucleotide sequences of representatives of the *Streptomyces* genus retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor in 1969 [19] and a phylogenetic tree was established using the neighbor-joining method of Saitou and Nei in 1987 [20]. The

topology of the tree was evaluated by bootstrap analysis using 1000 resamplings [21].

2.2. Production, extraction of bioactive compounds and anti-*A. tumefaciens* activity determination

The TN71 strain was selected based on its high antibacterial and antifungal activities against *A. tumefaciens*. All the antimicrobial production experiments were carried out in 1000 mL Erlenmeyer flasks with 100 ml of production medium prepared with different nutrients concentration according to the selected factorial design. The flasks were inoculated with 10 ml of spores at $10^7/L$ of *Streptomyces* strain TN71 and incubated on orbital shakers (250 rpm) for 4 days at 30 °C. The cell-free supernatant was extracted with ethyl acetate ($2 \times$) and the obtained organic extract was concentrated in vacuum to dryness. The crude extract was assayed in triplicates for their antibacterial activity against *A. tumefaciens* by disc diffusion method. *A. tumefaciens* was grown overnight in LB medium (NaCl 10 g/L, pancreatic digest of casein 10 g/L and yeast extract 5 g/L, pH 7.0) at 30 °C for 24 h, then diluted 1:100 in LB medium and incubated for 5 h under constant agitation on 200 rpm. Anti *A. tumefaciens* activity was determined by the agar diffusion test: a paper disk (8 mm \varnothing) was impregnated with 50 μ g of the corresponding sample and then laid on the surface of an agar plate containing 3 ml of top agar inseeded with 40 μ l of a 5 h-old culture of the corresponding microorganism. After 2 h at 4 °C, plates containing *A. tumefaciens* were incubated at 30 °C.

2.3. Selection of basic medium

Anti *A. tumefaciens* activity was determined upon growing of the TN71 strain on five different basal production media namely: (BM1) Bennett's medium: glucose 10 g/L, pancreatic digest of casein 2 g/L, yeast extract 1 g/L and beef extract 1 g/L, pH 7.0; (BM2) Czapek medium: sucrose 30 g/L, NaNO₃ 3 g/L, K₂HPO₄ 1 g/L, KCl 0.5 g/L, MgSO₄·7H₂O 0.5 g/L and FeSO₄·7H₂O 0.01 g/L pH 7.3; (BM3) GYM + S medium: starch 20 g/L, malt extract 10 g/L, CaCO₃ 4 g/L, glucose 4 g/L, and yeast extract 4 g/L, pH 7.2); (BM4) ISP medium 4: starch 10 g/L, CaCO₃ 2 g/L, (NH₄)₂SO₄ 2 g/L, K₂HPO₄ 1 g/L, MgSO₄·7H₂O 1 g/L, NaCl 1 g/L, FeSO₄·7H₂O 1 mg, MnCl₂·7H₂O 1 mg and ZnSO₄·7H₂O 1 mg, pH 7.2) and (BM5) Waksman's Glucose medium: glucose 10 g/L, peptone 5 g/L, beef extract 5 g/L and NaCl 5 g/L, pH 7.5) [22]. All the media (100 ml) were inoculated with 10 ml of the seed culture and incubated at 30 °C, 250 rpm for 4 days. After the incubation, crude extract from the culture broth was assayed against *A. tumefaciens*. The medium that showed high anti *A. tumefaciens* activity was selected for subsequent statistical optimization.

2.4. Screening of essential medium components using the PBD

The PBD was used to analyze important factors. Twelve experiments were conducted in duplicate to evaluate five factors. A total of five components were selected for the study, with each variable being represented at two levels, high (+) and low (–), as well as two dummy variables in twelve trials. The effect of each variable on the anti-*A. tumefaciens* activity was calculated and their significance was determined via Student's t-test using Minitab 15.0 version (Minitab Inc., PA, USA). The variables with confidence levels above 95% were considered to have significant effect on antimicrobial compound production and chosen for further optimization.

2.5. Box–Behnken design and optimization by RSM

The optimization of anti *A. tumefaciens* activity was carried out according RSM. The significant variables were optimized for enhanced anti *A. tumefaciens* activity by employing a Box–Behnken design [23]. Three variables (A: Starch; B: Malt extract; D: Glucose) were selected for

studying the effect and significance on anti- *A. tumefaciens* activity. These selected variables were analyzed at three levels low, medium, and high coded as -1, 0, and +1 in a total of twelve runs. The dummy variables were used to calculate the standard error. Each run was carried out with three replicates. The behavior of the system was explained by a second-order polynomial equation (Eq (1)).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the predicted response, β_0 is offset term β_i is linear effect, β_{ii} is squared effect, β_{ij} is interaction effect, and X_i is dimensionless coded value of independent variables under study. This design was used to evaluate: the main effects, interaction effects, and quadratic effects. It is also used to optimize the levels of parameters for enhancing anti *A. tumefaciens* activity. The statistical software (Minitab 15.0 version) was used for experimental design and data analysis. The statistical adequacy of the model was determined through analysis of variance (ANOVA). Three-dimensional response surface plots were drawn to illustrate the relationship between the responses and the experimental levels of each independent variable. An optimum level of the variables for maximum anti *A. tumefaciens* activity was determined by response optimizer tool of the software.

2.6. Artificial neural networks (ANN)

The data used for the optimization of anti *A. tumefaciens* activity from *Streptomyces* sp TN71 by RSM was also used for the optimization by ANN for comparison of two techniques. The selection of the input factors is the main aspect of neural network modelling [24]. The number of the hidden neurons depends on the character of the investigated problem. In fact, it has been argued that a network with a single hidden layer can be used to model any function [24]. Therefore, the used MLP and RBF neural network structures consisted of only one hidden layer. Thus, designing the ANN implies the selection of a satisfactory number of hidden neurons and suitable network organizations in concordance with type and nature of inputs (discrete, continuous, categorical, quantitative, etc). The determination coefficient (R^2) is used to control the accuracy of the forecast capacity of the constructed models. The training data set is applied to teach the ANN to find the global comprehensive model between its inputs and outputs. The test data are used to verify and confirm the predictive quality of the extended networks.

Thus, designing the ANN implies the selection of a satisfactory number of hidden neurons and suitable network organizations that would keep the inputs to the networks unchanged. The number of hidden neurons was optimized by reducing an error function that mapped the number of hidden nodes to the accuracy of the expanded networks. The Neural Network module of STATISTICA 8.0 software was used in modelling the ANN. Data were categorized into the two parts: training (80%), and testing (20%). In the network, there were seven inputs and one output, corresponding to medium ingredients and cellular yield, respectively. The hidden neurons were optimized by building various MLP and RBF ANNs with hidden nodes of one to thirty. Networks with hidden nodes greater than 30 were not developed due to the predictive capabilities decreasing as the number of intermediate units. The determination coefficient was used to choose the best model.

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) and Tukey's post hoc test was performed to determine significant differences between the responses using SPSS 19 statistical package (SPSS Ltd., Woking, UK). Means and standard errors were calculated. Differences among the mean values of the various responses were determined by the least significant difference test. A probability level of $P < 0.05$ was used in testing the statistical significance of all experimental data.

Table 1
Culture characteristics of strain TN71 on various media.

| Medium | Growth | Sporulation | Aerial mycelium | Substrate mycelium |
|---------------|----------|-------------|-----------------|--------------------|
| ISP medium 2 | Good | Good | White-grayish | Yellow-brown |
| ISP medium 3 | Good | Good | White | Pale yellow |
| ISP medium 4 | Good | Good | White | Pale yellow |
| ISP medium 5 | Good | Good | White-grayish | Yellow-brown |
| Bennett agar | Good | Good | White-grayish | Pale yellow |
| Nutrient agar | Moderate | Moderate | White-grayish | Yellow-brown |

3. Results and discussion

3.1. Morphology characteristics

Strain TN71 is a Gram-positive bacterium. Morphological observation of the 7–15 days old culture revealed that this strain showed good sporulation and growth on ISP medium 2, ISP medium 3, ISP medium 4, ISP medium 5 and Bennett media. Aerial mycelium varied from white to grayish-white in all tested media and the substrate hyphae varied from pale yellow to yellow-brown (Table 1).

3.2. Phylogenetic analysis

The nucleotide sequence of the whole 16S rRNA gene (1439 pb) of TN71 strain has been assigned in the GenBank (EMBL) under accession number MG198761.

This sequence was aligned with those of *Streptomyces* reference species available in the GenBank database, which confirmed that the isolate TN71 belonged to the genus *Streptomyces*. Comparing 16S rRNA gene sequences, TN71 was mostly related with *Streptomyces cyaneus* NRRL B-2296^T (99.79%) [13], *Streptomyces violarius* NBRC 13104^T (99.71%) [25], *Streptomyces speibonae* ATCC BAA411^T (99.67%) [26] and *Streptomyces albogriseolus* NRRL B-1305^T (99.64%) [27] (Fig. 1).

3.3. Physiological characteristics

The differential characteristics of strain TN71 and its closest neighbors in the genus *Streptomyces* are given in Table 2. Melanoid pigments were produced on ISP medium 6 and ISP medium 7 for TN71, *S. cyaneus* NRRL B-2296^T and *S. albogriseolus* NRRL B-1305^T but not produced for *S. violarius* NBRC 13104^T. The five strains of *Streptomyces* share some physiological characters like utilization of sole carbon and nitrogen sources: D-fructose, D + galactose, Glycerol and D-mannitol (sole carbon sources) and L-histidine, L-serine and L-valine (sole nitrogen sources). The differences consisted of the degradation of inositol, maltose, rhamnose, raffinose, sucrose and D-xylose (sole carbon sources), L-arginine, L-cysteine, L-hydroxyproline, L-methionine, L-phenylalanine and L-threonine (sole nitrogen sources). Growth of the three *Streptomyces* strains (TN71, NRRL B-2296^T and NRRL B-1305^T) was observed at a wide range of temperatures (30–45 °C) and concerning the TN71 strain, the optimal temperature of growth was ranged between 30 and 35 °C. The two strains TN71 and *Streptomyces albogriseolus* NRRL B-1305^T were capable to grow in the presence of 7% NaCl (Table 2).

3.4. Selection of basal medium

As compiled in Table 3, the crude extract showed varying degrees of anti *A. tumefaciens* activity. The inhibition zones were in the range of (11.00 ± 1.00–22.33 ± 0.58 mm). Among the five different growth media, the highest activity ($P < 0.05$) was observed in GYM + S (BM3) containing 20 g of starch, 10 g of malt extract, 4 g of CaCO₃, 4 g of glucose and 4 g of yeast extract (Table 3). Therefore, BM3 was selected for further optimization study using statistical approaches.

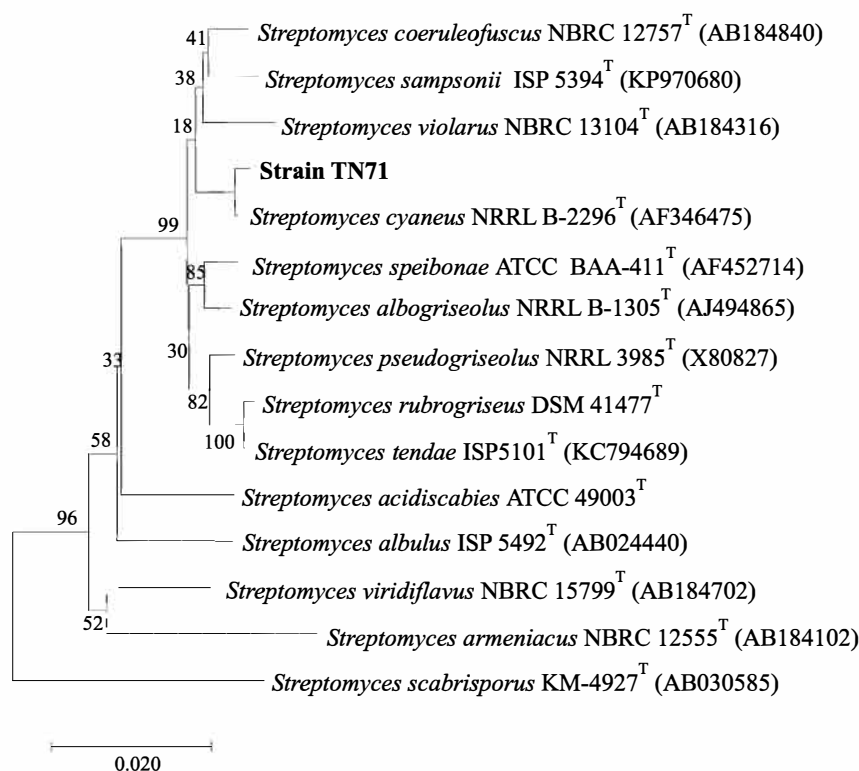


Fig. 1. Phylogenetic trees derived from 16S rDNA sequence of *Streptomyces* sp. TN71 strain. All the sequences used here were from *Streptomyces* type strains. Genbank accession numbers for all species are given in parenthesis. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. Bar 0.020 substitutions per nucleotide position.

Table 2
Phenotypic properties of TN71 strain compared with closely related species of the *Streptomyces* species.

| Characteristics | 1 | 2 | 3 | 4 | 5 |
|---------------------------------|--------------------|--------------------|----------|--------------|--------------------|
| Melanoid pigments production on | | | | | |
| ISP medium 6 and 7 | ISP medium 6 and 7 | ISP medium 6 and 7 | - | ISP medium 6 | ISP medium 6 and 7 |
| Growth on sole carbon sources | | | | | |
| L-arabinose | + | + | - | + | + |
| D-fructose | + | + | + | + | + |
| D + galactose | + | + | + | + | + |
| D-glucose | + | + | + | + | + |
| Glycerol | + | + | + | + | + |
| iso-inositol | - | + | + | + | + |
| Maltose | + | - | - | + | + |
| D-mannitol | + | + | + | + | + |
| Rhamnose | - | + | + | + | + |
| Raffinose | - | + | - | + | - |
| Sucrose | + | + | - | + | - |
| D-xylose | + | + | + | + | + |
| Growth on sole nitrogen sources | | | | | |
| L-arginine | + | - | - | + | + |
| L-cysteine | + | ND | ND | + | + |
| L-histidine | + | + | + | + | + |
| L-hydroxyproline | - | + | + | - | - |
| L-methionine | + | - | - | + | + |
| L-phenylalanine | + | - | + | + | - |
| L-serine | + | + | + | + | + |
| L-threonine | - | - | + | + | + |
| L-valine | + | + | + | + | + |
| NaCl Tolerance | Until 7% | Until 6% | Until 5% | Until 6% | Until 10% |
| Growth at 45 °C | + | + | - | - | + |

Taxa: 1, *Streptomyces* sp. strain TN71; 2, *Streptomyces cyaneus* NRRL B-2296^T (Waksman and Lechevalier 1953); 3, *Streptomyces violarius* NBRC 13104^T (Pridham 1970); 4, *Streptomyces speibonae* ATCC BAA-411^T (Meyers et al., 2003); 5, *Streptomyces albogriseolus* NRRL B-1305^T (Benedict et al., 1954); + positive, - negative.

Table 3
Effect of five different production media on anti *A. tumefaciens* activity in *Streptomyces* sp. TN71.

| Basal medium | <i>A. Tumefaciens</i> (mm) |
|--------------|----------------------------|
| BM1 | 13.00 ± 1.00 ^{ab} |
| BM2 | 18.50 ± 2.00 ^c |
| BM3 | 22.33 ± 0.58 ^d |
| BM4 | 15.33 ± 1.53 ^b |
| BM5 | 11.00 ± 1.00 ^a |

± : Standard Error.
a-d: Averages with different letters in the same column, for each parameter, are different ($P < 0.05$).

3.5. Selection of significant media components by the PBD

In view of media components significance and their optimum levels for the secondary metabolite production, we have attempted to optimize medium components for enhanced anti *A. tumefaciens* activity of *Streptomyces* sp. strain TN71. The significant media components for enhancing antibacterial compound production were screened and selected using PBD as shown in Table 4a, b. In fact, five variables supposed to affect anti-*A. tumefaciens* activity were evaluated under twelve experiments for the PBD. Table 4a shows the responses obtained in terms of anti *A. tumefaciens* activity. The responses were statistically evaluated and the variables with P value less than 0.05 (confidence levels above 95%) were considered to have a significant effect on antimicrobial activity. The regression coefficients and determination coefficients (R^2) for the linear regression model of the antimicrobial activity were represented in Table 4b. It should be noted that R^2 value is considered as a principal criterion for the predictive success of the models. Multivariate models capable of assessing large number of variables and interrelations are therefore, more successful in defining and predicting biological processes such as antimicrobial activity. In our study, the model was highly significant ($P < 0.05$), $R^2 = 0.9309$, meaning that 93.09% of the total variability in the response could be

Table 4a
PBD and the experimental response obtained for *Streptomyces* TN71.

| Trials | Variables | | | | | anti <i>A. tumefaciens</i> activity (mm) |
|--------|-----------|-------|------|------|------|--|
| | A | B | C | D | E | |
| 1 | +(30) | +(15) | -(2) | +(6) | +(6) | 33.33 ± 1.52 ^h |
| 2 | -(10) | -(5) | +(6) | +(6) | +(6) | 22.33 ± 0.75 ^d |
| 3 | -(10) | +(15) | -(2) | -(2) | -(2) | 17.50 ± 0.50 ^b |
| 4 | -(10) | -(5) | -(2) | +(6) | +(6) | 19.66 ± 2.08 ^{bc} |
| 5 | +(30) | -(5) | -(2) | -(2) | +(6) | 18.50 ± 1.77 ^b |
| 6 | +(30) | -(5) | +(6) | +(6) | -(2) | 25.00 ± 3.00 ^e |
| 7 | -(10) | +(15) | +(6) | +(6) | -(2) | 28.00 ± 1.00 ^f |
| 8 | -(10) | +(15) | +(6) | -(2) | +(6) | 21.86 ± 0.57 ^{cd} |
| 9 | +(30) | +(15) | +(6) | -(2) | +(6) | 31.36 ± 1.52 ^g |
| 10 | +(30) | +(15) | -(2) | +(6) | -(2) | 29.16 ± 0.76 ^f |
| 11 | -(10) | -(5) | -(2) | -(2) | -(2) | 15.00 ± 1.00 ^a |
| 12 | +(30) | -(5) | +(6) | -(2) | -(2) | 20.33 ± 2.08 ^c |

A: Starch; B: Malt extract; C: CaCO₃; D: Glucose; E: Yeast extract.
± : Standard Error.

a–h: Averages with different letters in the same column, for each parameter, are different ($P < 0.05$).

Table 4b
Effect estimates for anti-*A. tumefaciens* activity from the result of the PBD.

| | <i>A. tumefaciens</i> | | | |
|----------------|-----------------------------------|-------|-------|-------------|
| | Effect | T | P | Coefficient |
| Constant | | 38.12 | 0.000 | 23.417 |
| A | 5.5 | 4.48 | 0.001 | 2.750 |
| B | 6.833 | 5.56 | 0.041 | 3.417 |
| C | 2.5 | 2.03 | 0.188 | 1.250 |
| D | 5.833 | 4.75 | 0.003 | 2.917 |
| E | 2.167 | 1.76 | 0.058 | 1.083 |
| R ² | R-Sq = 93.09%; R-Sq(adj) = 87.32% | | | |

A: Starch; B: Malt extract; C: CaCO₃; D: Glucose; E: Yeast extract.

explained by this model. The P values of the important variables in the PBD as given below were the most significant variables affecting anti-*A. tumefaciens* activity production by *Streptomyces* sp. strain TN71 (Table 4b): starch ($P = 0.001$), glucose ($P = 0.003$), malt extract ($P = 0.041$) and yeast extract ($P = 0.058$). Moita et al. in 2005 reported that effects with $P < 0.05$ are highly significant and P -value < 0.1

Table 5a
Box-Behnken design and ANN models of variables (in coded levels), with anti-*A. tumefaciens* responses.

| Runs | Coded values | | | Experimental antibiotic activity (mm ± s.e.m) | Predicted antibiotic activity (mm) RSM | Predicted antibiotic activity (mm) ANN |
|------|--------------|-------|------|---|--|--|
| | A | B | D | | | |
| 1 | 0(20) | +(15) | +(6) | 18.26 ± 1.53 ^a | 16.57 ^a | 17.95 ^a |
| 2 | 0(20) | +(15) | -(2) | 21.06 ± 2.10 ^{bc} | 21.32 ^{cd} | 22.34 ^c |
| 3 | +(30) | 0(10) | -(2) | 29.83 ± 3.20 ^g | 28.92 ^f | 29.66 ^f |
| 4 | 0(20) | -(5) | +(6) | 21.06 ± 0.50 ^{bc} | 20.77 ^c | 21.81 ^{bc} |
| 5 | -(10) | 0(10) | -(2) | 21.03 ± 0.70 ^{bc} | 20.42 ^c | 21.58 ^{bc} |
| 6 | -(10) | +(15) | 0(4) | 18.06 ± 1.17 ^a | 18.44 ^b | 18.24 ^a |
| 7 | 0(20) | -(5) | -(2) | 22.13 ± 2.64 ^c | 23.52 ^d | 24.38 ^d |
| 8 | +(30) | 0(10) | +(6) | 22.20 ± 2.22 ^c | 22.67 ^d | 22.79 ^c |
| 9 | -(10) | -(5) | 0(4) | 20.13 ± 2.05 ^b | 19.14 ^{bc} | 18.63 ^a |
| 10 | +(30) | +(15) | 0(4) | 20.93 ± 2.28 ^b | 21.94 ^{cd} | 20.15 ^b |
| 11 | +(30) | -(5) | 0(4) | 28.30 ± 0.80 ^f | 27.64 ^{ef} | 27.53 ^e |
| 12 | -(10) | 0(10) | +(6) | 17.83 ± 2.92 ^a | 19.17 ^{bc} | 18.91 ^a |
| 13 | 0(20) | 0(10) | 0(4) | 23.86 ± 1.80 ^{cd} | 26.04 ^e | 24.85 ^d |
| 14 | 0(20) | 0(10) | 0(4) | 26.83 ± 0.41 ^c | 26.04 ^e | 24.85 ^d |
| 15 | 0(20) | 0(10) | 0(4) | 24.63 ± 0.85 ^d | 26.04 ^e | 24.85 ^d |
| 16 | 0(20) | 0(10) | 0(4) | 25.12 ± 0.46 ^d | 26.04 ^e | 24.85 ^d |
| 17 | 0(20) | 0(10) | 0(4) | 24.20 ± 0.72 ^d | 26.04 ^e | 24.85 ^d |

Variables: A: Starch; B: Malt extract; D: Glucose.

± : Standard Error.

a–g: Averages with different letters in the same column, for each parameter, are different ($P < 0.05$).

Table 5b
Regression coefficients and their significance for response surface model of anti-*A. tumefaciens* activity.

| | <i>A. tumefaciens</i> | | |
|----------------|---|--------|-------------|
| | P | T | Coefficient |
| Constant | 0.175 | -1.58 | -11 |
| B | 0.009 | 4.19 | 2.975 |
| D | 0.03 | 2.99 | 5.312 |
| A | 0.02 | 3.38 | 1.2 |
| B ² | 0.007 | -4.47 | -0.13 |
| D ² | 0.027 | -3.1 | -0.0562 |
| A ² | 0.227 | -1.38 | -0.01 |
| B × D | 0.506 | -0.72 | -0.05 |
| B × A | 0.133 | -1.790 | -0.025 |
| D × A | 0.133 | -1.790 | -0.062 |
| R ² | R ² = 95.12%; R ² _{adj} = 86.33% | | |

shows a moderate significant effect [28]. Hence, from the experimental data, the three variables listed above could clearly affect the anti-*A. tumefaciens* activity. Malt extract, glucose and starch have a positive effect on antimicrobial activity. Considering the results displayed in Table 4b and after exclusion of the insignificant model terms ($P > 0.05$) the reduced polynomial Eq. (2) may be written as follows:

$$\text{Anti-}A. \text{ tumefaciens activity} = 23.417 + 2.75 \times A + 3.417 \times B + 2.917 \times D \quad (2)$$

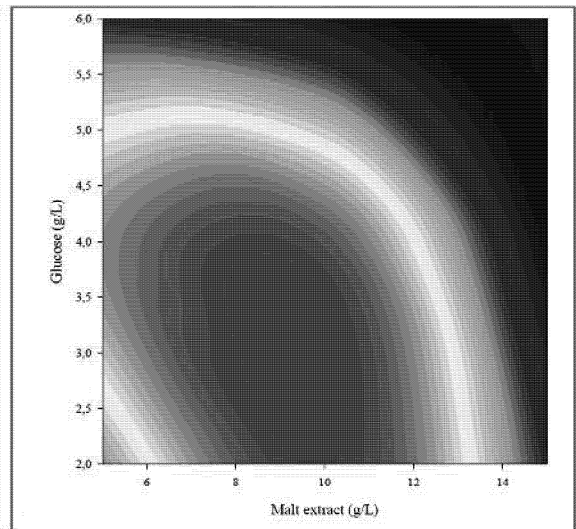
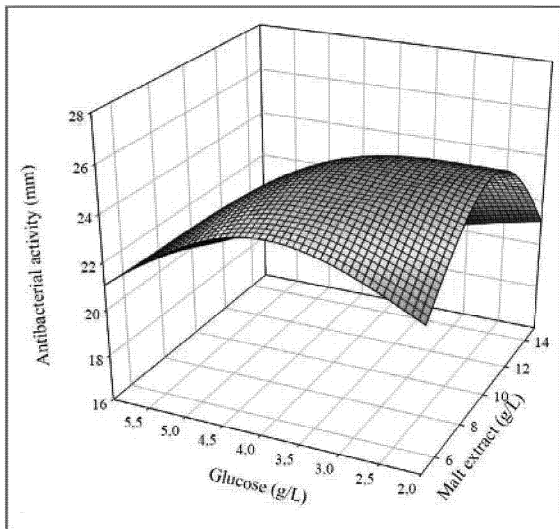
Where A: Starch; B: Malt extract; D: Glucose.

3.6. Optimization of selected media components by the Box-Behnken design

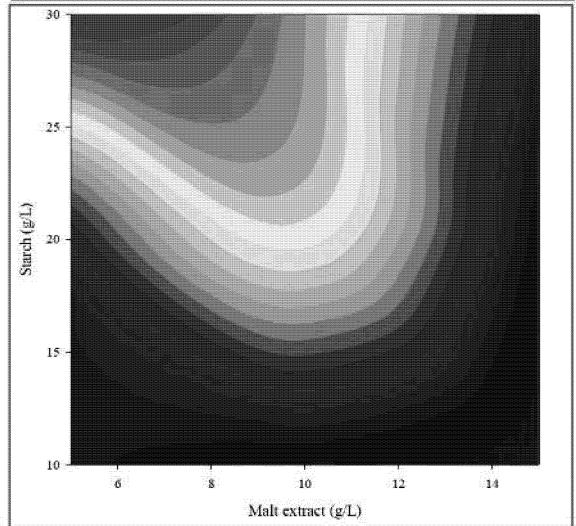
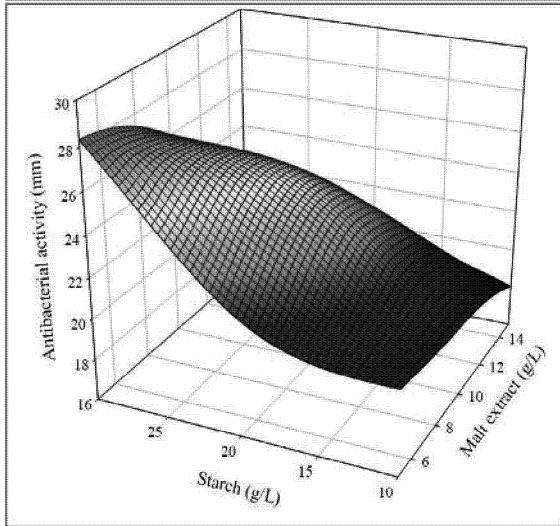
Based on the selection of variables by PBD, a Box-Behnken experimental plan was carried out for variables that affected significantly anti-*A. tumefaciens* activity. Table 5a shows the real and the coded values of the levels of variables selected in the Box-Behnken design. The forecasted and observed values of the response are described in Table 5a. A multiple regression analysis was applied to the experimental data, and a second order polynomial equation was found to explain the anti *A. tumefaciens* activity by *Streptomyces* sp. strain TN71 (Eq. (3)).

$$\text{Anti-}A. \text{ tumefaciens activity} = -11 + 2.98 \times B + 5.31 \times D + 1.2 \times A -$$

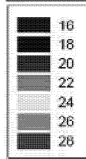
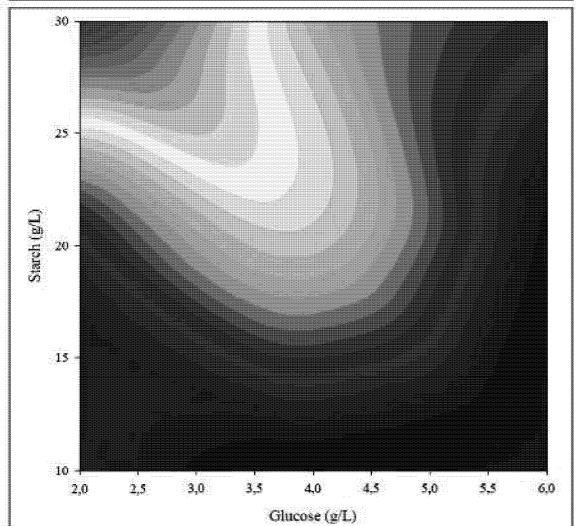
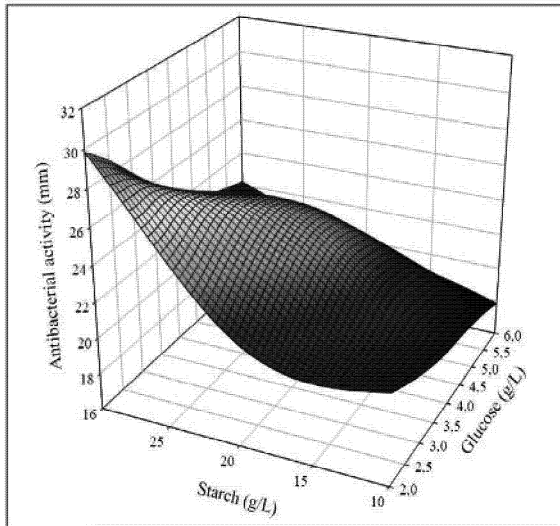
a



b



c



(caption on next page)

Fig. 2. (a) 3D response surface curve (left) for anti *A. tumefaciens* activity affected by glucose and malt extract, when starch was maintained at 20 g/L. Two-dimensional contour plot (right) showing anti *A. tumefaciens* activity in response to varying of glucose and malt extract; (b) 3D response surface curve (left) for anti *A. tumefaciens* activity affected by starch and malt extract, when glucose was maintained at 4 g/L. Two-dimensional contour plot (right) showing anti *A. tumefaciens* activity in response to varying of starch and malt extract; (c) 3D response surface curve (left) for anti *A. tumefaciens* activity affected by starch and glucose, when malt extract was maintained at 10 g/L. Two-dimensional contour plot (right) showing anti *A. tumefaciens* activity in response to varying of starch and glucose.

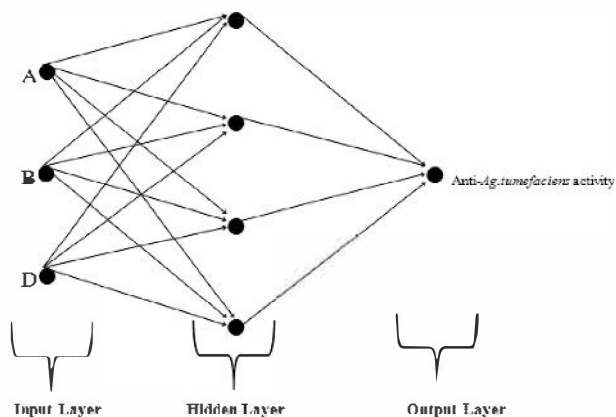


Fig. 3. Graphical representation of MLP neural network containing three components.

$$0.13 \times B^2 - 0.562 \times D^2 - 0.01 \times A^2 - 0.05 \times B \times D - 0.25 \times B \times A - 0.0625 \times D \times A \quad (3)$$

The experimental results revealed that this polynomial equation could satisfactorily explain the effects of the most significant variables concentration in the anti *A. tumefaciens* activity by *Streptomyces* sp. strain TN71. The robustness of the model was determined by calculating the determination coefficient R^2 (0.9512), which suggested that it is a reliable model and that it is able to explain more than 95.12% of the total variations. Only 13.67% of the total variation of anti- *A. tumefaciens* activity was not explained by the model. The relatively high adjusted determination coefficient ($R_{adj}^2 = 0.8633$) accounts for the significance of the model (Table 5b). Montgomery in 2001 reported that both the high adjusted R^2 value and the close to zero P -value in the Analysis Of Variance (ANOVA) show that this model has a satisfactory

goodness of fit. In order to gain a better understanding of the variables effects on anti- *A. tumefaciens* activity, the predicted model was presented as 3D response surface graphs (Fig. 2). The 3D plots clearly showed that the maximum anti *A. tumefaciens* activity should occur with higher level of starch, moderate level of malt extract and lower level of glucose (Fig. 2). Raytapadar and Paul in 2001 proposed glucose and starch for maximum antibiotic production from *Streptomyces aburaviensis* 1DA-28 [29]. The optimum concentration of glucose and starch were found to be 2% and 4% (w/v), respectively. Similarly, the results of Singh et al. in 2009 and Vasavada et al. in 2006 showed that anti-*Staphylococcus aureus* activities produced by *Streptomyces tanshiensis* strain A2D and *Streptomyces sannanensis* strain RJT-1 respectively, were higher in medium having glucose at 1% (w/v) [30,31]. Secondary metabolites production in actinomycetes is often stimulated by slowly-assimilated complex carbohydrates such as soluble starch, but is suppressed by rapidly-utilized carbon sources such as presence of glucose [32]. In fact, high concentration usually inhibits the biosynthesis of many antibiotics in *Streptomyces* [33–36]. A possible explanation of this phenomenon is that glucose may cause catabolic repression, in which the production of enzymes for secondary metabolites biosynthesis might be inhibited [32,37]. An example of catabolic repression of secondary metabolism in actinomycetes is that of actinomycin synthesis by *Streptomyces antibioticus* when more glucose is added to the media [37]. In a medium containing glucose in addition to a more slowly utilized carbon source, glucose usually is used first in the absence of antibiotic production. After glucose is depleted, the second carbon source is then used for antibiotic biosynthesis [47]. For the majority of *Streptomyces*, the preferred carbon source is starch, especially for the production of secondary metabolites [38]. In concern to malt extract, considered as the principal provider of nitrogenous source in studied culture medium, the concentration is estimated about 5.06 g/L [39,40]. In the present study, starch at 19.49 g/L and glucose at 2.07 g/L enhanced the anti-*A. tumefaciens* activity. Thus, it is not

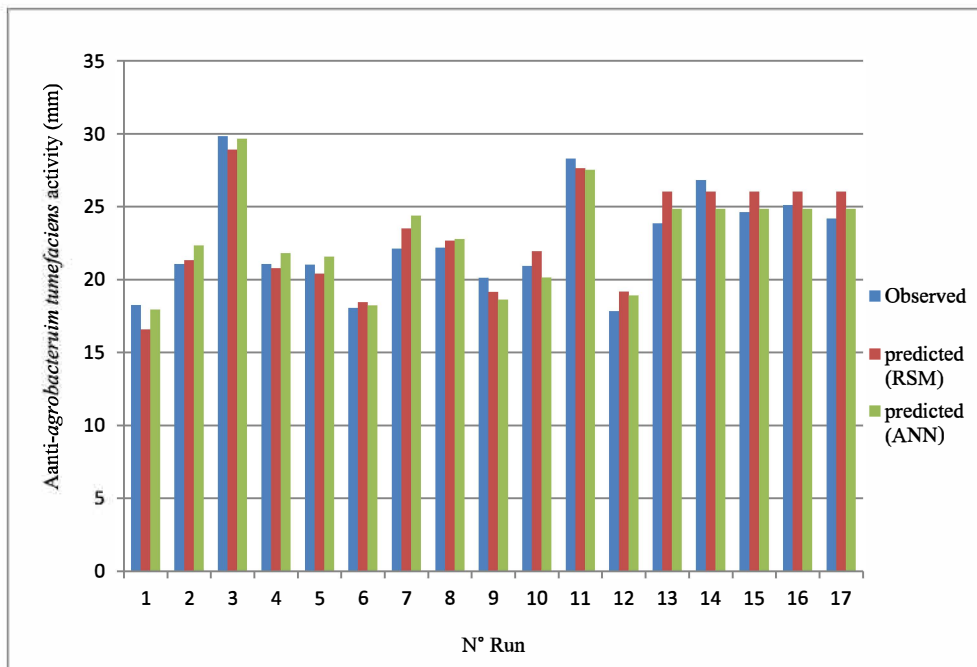


Fig. 4. Comparison between observed and predicted anti-*A. tumefaciens* activity from the two models (RSM and ANN).

surprising that corn starch is a significant factor, as shown by PBD results.

3.7. Generation of ANNs models for anti-*A. tumefaciens* activity

To develop a model based on Neural Network performance for anti-*A. tumefaciens* activity prediction, several ANN networks in MLP and RBF structure were constructed and tested in order to determine the optimum number of neurons, hidden layers and transfer functions. In fact, establishing an adequate structure with the suitable number of hidden layers, and neurons is important, since a larger number could result in over-fitting, while a smaller number, may not process the data sufficiently. Several MLP and RBF networks were developed and trained using the learning data set and then they were validated with the test data set. The optimal obtained network model with maximum coefficient of determination (R^2) was selected. In this study, ANNs were used to forecast anti *A. tumefaciens* activity, in fact, after several model runs, the best structure with the highest R^2 was depicted. This structure constitutes the selected ANN model for the anti-*A. tumefaciens* activity prediction. It is an MLP with three layer perceptron described as follows: one input layer with seven input variables, one hidden layer with four neurons and one output layer with one output variable (Fig. 3). The results of the model trained created high determination coefficients R^2 ($R^2 = 96.64\%$ and 98.69% for training and test respectively). The predicted and observed anti-*Agrobacterium tumefaciens* activity is shown in Table 5a.

In this work, an attempt was made to analyse and compare RSM and ANN including MLP and RBF models to develop transfer function for predicting antibacterial yields by using medium ingredients. The statistical prediction performances of used models were measured in terms of determination coefficient (R^2). Hence, the results obtained from the RSM models indicated that the measured determination coefficient between the observed and the predicted data were acceptable with a determination coefficient higher than 70% for both anti-*A. tumefaciens* activity. Furthermore, ANN models showed higher accuracy ($R_{\text{test}}^2 = 98.69$) when compared with RSM models ($R_{\text{adj}}^2 = 86.33$).

The comparison between experimental with predicted outputs from ANN and RSM was shown in Fig. 4. The two models were well fitted to experimental data. As shown, the target (predicted) and output data (experimental) are in a good agreement indicating the high accuracy of the RSM and ANNs developed models. The ANN model improved the adjustments in comparison with the RSM model. In fact, the ANN model presents an improvement of 12.36% in terms of determination coefficients of anti-*A. tumefaciens* activity (Fig. 4).

3.8. Validation of the optimized condition

On the basis of numerical optimization of the predicted models, the maximum anti-*A. tumefaciens* activity was 28.92 mm, when the optimal values of test factors were 19.49 g/L for starch, 5.06 g/L for malt extract and 2.07 g/L for glucose (Table 5a). To verify the predicted results, validation experiment was performed in triplicate tests. An increasing of 29.51% was shown between the inhibition of original medium and the optimized medium (22.33 ± 0.58 mm) for anti-*A. tumefaciens* (Table 3). Validating and predicted values are basically the same, indicating that the model is reasonable and effective.

4. Conclusion

The practicability of statistical/artificial intelligence techniques, such as response surface methodology (RSM) and artificial neural network (ANN) have been tested to optimize the medium composition for high anti-*A. tumefaciens* activity from *Streptomyces* sp. TN71. To explore the biological control of *Streptomyces* sp. TN71 strain against *A. tumefaciens*, RSM and ANN were used to derive a model for interaction effects of medium components: starch, malt extract, CaCO_3 , glucose and

yeast extract. This study proved that statistical experimental designs offer an efficient and feasible approach for antimicrobial medium optimization. A maximum anti-*A. tumefaciens* activity of 29.11 ± 2.11 mm was achieved with the following optimized factors: starch 19.49 g/L, malt extract 5.06 g/L, and glucose 2.07 g/L. Validation experiments were also carried out to verify the adequacy and the accuracy of the RSM models, and results showed that the predicted value agreed with the experimental values well, and an increasing of 30.36% was shown between the inhibition of original medium and the optimized medium for anti-*A. tumefaciens* activity. It is obvious that MLP and RBF architecture networks were most suitable for anti-*A. tumefaciens* activity. The architecture networks are strictly dependent on the data types. Moreover, the ANN model improved the adjustments in comparison with the RSM models: the ANN models present an improvement of 12.36% in terms of determination coefficients of anti-*A. tumefaciens* activity. The results also give a basis for further study with large scale fermentation for production of biological control based on *Streptomyces* sp. TN71 antibacterial metabolites.

Conflicts of interest

The authors have declared that there are no conflicts of interest.

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