RESPONSE SURFACE METHODOLOGY-ARTIFICIAL NEURAL NETWORK-BASED OPTIMIZATION AND STRAIN IMPROVEMENT OF CELLULASE PRODUCTION BY *STREPTOMYCES SP.*

METODOLOGIA DE SUPERFÍCIE DE RESPOSTA - OTIMIZAÇÃO BASEADA EM REDES NEURAIS ARTIFICIAIS E MELHORIA DE ESTIRPES NA PRODUÇÃO DE CELULASES POR STREPTOMYCES SP.

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ABSTRACT: Thirty-seven different colonies were isolated from decomposing logs of textile industries. From among these, a thermotolerant, gram-positive, filamentous soil bacteria *Streptomyces durhamensis* vs15 was selected and screened for cellulase production. The strain showed clear zone formation on the CMC agar plate after Gram's iodine staining. *Streptomyces durhamensis* vs15 was further confirmed for cellulase production by estimating the reducing sugars through the dinitrosalicylic acid (DNS) method. The activity was enhanced by sequential mutagenesis using three mutagens of ultraviolet irradiation (UV), N methyl-N'-nitro-N-nitrosoguanidine (NTG), and Ethyl methanesulfonate (EMS). After mutagenesis, the cellulase activity of GC23 (mutant) was improved to 1.86-fold compared to the wild strain (vs15). Optimal conditions for the production of cellulase by the GC 23 strain were evaluated using Response Surface Methodology (RSM) and Artificial Neural Network (ANN). The effects of pH, temperature, duration of incubation, and substrate concentration on cellulase production were evaluated. Optimal conditions for the production of cellulase enzyme using Carboxymethyl cellulose as a substrate are 55 ºC of temperature, pH of 5.0, and incubation for 40 h. The cellulase activity of the mutant *Streptomyces durhamensis* GC23 was further optimized to 2-fold of the activity of the wild type by RSM and ANN.

KEYWORDS: *Streptomyces durhamensis*. Mutagenesis. Response surface methodology. Artificial neural network. Carboxymethyl Cellulose.

INTRODUCTION

With the increasing fuel demand these days, the world is in search of alternate sources of bioenergy. The abundant "lignocellulosic biomass" can be a prime source for the production of bioethanol. In addition to fuel generation, it can also be used to produce other valued products like organic acids, fermentable sugars, drink softeners, solvents, etc. Lignocellulosic biomass comprises of three major components of cellulose, hemicellulose, [illegible] 1, 4 glycoside bonds act as a major constituent of all plant material including agricultural wastes. Cellulose is hydrolyzed to glucose units by the action of three cellulolytic enzymes: cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). These enzymes are used in the laundry, paper, pulp, textile industries (BHAT, 2000).

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Streptomyces are gram-positive, filamentous soil bacteria. They are a source for a large variety of antibiotics and enzymes of commercial or academic value. Different strains of *Streptomyces* are found to be secreting a large variety of enzymes that hydrolyze hemicellulose, cellulose, and lignin (JANG; CHEN, 2003).

Strain improvement has been achieved through selection, mutation, or genetic recombination(PAREKH; VINCI; STROBEL, 2000). In cellulase production, mutation via mutagenic agents like ultraviolet (UV) rays, gamma radiation, X-rays, ethyl methanesulfonate (EMS), [illegible] soguanidine' (NTG) [illegible] for their efficiency (SANGKHARAK; VANGSIRIKUL; JANTHACHAT, 2012). Treatment of *Aspergillus* sp with γ-irradiation of $Co^{60,}$ ultraviolet and N-methyl-N'-nitro-N nitrosoguanidine has been used for enhanced cellulase production (VU; PHAM; KIM, 2009). The simultaneous treatment of a *Cellulomonas* sp. TSU-03 with UV irradiation and NTG improved cellulase production.

In microorganisms, cellulase production can be significantly influenced by nutritional and

physical parameters such as incubation period, pH, temperature, and agitation speed (DAS et al., 2013). Optimization by classical methods such as one-at-a-time or mathematical methods may lead to unreliable results and inaccurate conclusions, while the statistical approach of Response Surface Methodology (RSM) is more reliable. It can study many variables simultaneously from a low number of observations, reducing time and cost (BEZERRA et al., 2008; DEEPAK et al., 2008). Though RSM is frequently used for optimization, it has some limitations. By second order equation, all the changes happening in the process are hard to explain (BAŞ; BOYACı, 2007). Recently, artificial neural networks (ANN) showed a significantly higher simulation and estimation capabilities than RSM.

In this study, potential cellulolytic strains from decomposing logs of textile industries were isolated. A promising cellulase producing actinomycete strain designated as *Streptomyces durhamensis vs*15 was selected to improve the cellulase production. Strain improvement through mutation as well as by optimization of physical parameters such as incubation period, pH, incubation temperature, and substrate concentration were carried out by RSM and validation through ANN.

MATERIAL AND METHODS

Sample collection

Samples were collected from decomposing logs of textile industries of Surat, India. Isolation was performed on CMC agar plates (DICKERMAN; STARR, 1951). The inoculated plates were incubated at 37 ºC for 2 days. After 2 days of incubation at 37 ºC, the diameters of clear zones obtained by flooding the plates with Gram's iodine were measured iodine (KASANA et al., 2008). Cellulase activity was quantified by estimating the reducing sugars liberated by each isolate through the dinitrosalicylic acid (DNS) method (MILLER, 1959). For further applications, the sample was stored at 4 ºC, in CMC agar slants.

Cellulase production and assay

Cellulases were produced in submerged fermentation, carried out in 250 ml Erlenmeyer flasks containing 100 ml of CMC broth (DICKERMAN; STARR, 1951). The media were then incubated at 37° C in an orbital shaker set at 150 rpm for 2 days. Fermentations in triplicates were carried out for each culture medium. At the end of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4 ºC, and the clear supernatant was subjected to further studies. The enzyme assay was performed according to the method by Ghose (1987). The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 2.0% carboxymethyl cellulose solution. The enzyme mixture was incubated at 50 ºC for 30min. Dinitrosalicylic acid reagent (DNS; 3 ml) was added to each test tube. The reaction tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of the tubes were diluted to 20 ml with distilled water. The absorbance was read at 540 nm using a UV-Visible spectrophotometer (HALO DB20 UV-Visible double beam spectrophotometer). One cellulase unit is defined as the amount of enzyme that liberates reducing sugar at the rate of 1μmol/min under the standard assay conditions of supernatant solution. Total protein was estimated by the Bradford method using bovine serum albumin as the standard(BRADFORD, 1976).

Molecular Characterization

*Streptomyces durhamensis*vs15 was identified by 16S rRNA gene sequencing. Isolation of genomic DNA and amplification by 16S rRNA was carried out by Macrogen, South Korea, and the sequence obtained was deposited in the GenBank database.

Strain improvement by mutagenesis and screening

The strain *Streptomyces durhamensis* vs15 was grown in 10 ml of CMC broth at 37 ºC for 2 days. Mutagenesis was done with N-methyl-N–nitro-Nitrosoguanidine' (NTG), Ethyl methanesulfonate (EMS), and ultraviolet rays (UV). In treatment 1, cells were irradiated with UV (Philips 30W G30 TB) at a wavelength of 360 nm at a distance of 35 cm for (1-15 min). In treatment 2, cells of vs15 were selected for exposure to different concentrations (0.620-1.48 mM) of EMS for an hour. In treatment 3, mutagenesis with (2-5 μg) grains of NTG placed at the center of the plate was done. The lawn around the inhibition zone was scrapped after 24h incubation and cultivated in CMC broth for 8 h (YU et al., 2008). In treatment 4, the cells were exposed to a combination of 0.931 mM of EMS for an hour and UV irradiation for 5 min. In treatment 5, the treatment was done with a combination of 50-70 μg/ml of NTG for 30 min and UV irradiation for 3 min; the treated cells (0.1 ml) were spread on CMC media containing 2.5% (w/v) Carboxymethyl cellulose (CMC) and incubated at 37°C for 2 days. The selection of mutants was carried out based on the presence of larger halos

around the colonies and/or by faster-growing colonies. The mutants with high cellulase production were selected for further optimization for cellulase production.

Optimization of culture conditions by response surface methodology

Streptomyces durhamensis vs15 was grown on CMC medium broth at 37 ºC and 150 rpm for 2 days. Five independent variables factors, namely pH, temperature, substrate concentration, incubation time, and rate of agitation were selected for the one-factor-at-a-time method. The influences of each individual variable on cellulase yield were analyzed. Based on the results of the one-factor method, the critical factors are chosen for further optimization by Response surface methodology (RSM).

Using central composite design (CCD), each factor was analyzed at 5 different levels –α, -1, 0, +1.+α representing relatively low, low, center, high, relatively high respectively (SHARMA et al., 2007). The maximum and minimum values of 4 variables are shown in Table 1. According to central composite design for 4 factors, 30 runs were obtained for one responsible factor i.e., cellulase yield.

The experimental data were analyzed using a second-order polynomial equation,

$$\mathbf{Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j}$$

where Yi is the predicted response, βi is the linear coefficient, βii is the squared coefficient, βij is the interaction coefficient, xi is the independent effect, xi^2 is the squared effect, and x_ix_j is the interaction effect.

F-test was used to evaluate the model equation while ANOVA (analysis of variance) was used to analyze the statistical parameters. The fitness of the model polynomial equation was given by the R^2 regression correlation and the relations between responses in the design were displayed as three-dimensional plots.

Table 1. Process variables employed in the study of central composite design (CCD) optimization.

Process variables	-2	-1	0	1	2
Ph	3.5	4	4.5	5	5.5
Temperature	40 ^{0}C	45 ^{0}C	50 ^{0}C	55 ^{0}C	60 ^{0}C
Substrate Concentration	0.5%	1%	1.5%	2%	2.5%
Incubation	16 h	24 h	32 h	40 h	48 h

Artificial neural network

In order to improve the cellulase production, culture conditions and the concentration of substrate are the important factors to study. The design expert Response surface model was employed in the study to identify the culture conditions best suitable for the production of cellulase. After using the RSM model for the optimization of cellulase production, the experimental data were further used for testing and validation by the ANN. In the present study, the Multilayer perceptron (RAO et al., 2010) model of an artificial neural network is employed. It uses feed-forward architecture and works on the backpropagation algorithm (MARAN; MANIKANDAN; MEKALA, 2013). A three-layered model which includes inner, hidden, and output layers were used in the study. The top architecture of the feed-forward three-layered neural network is shown in Figure 1.

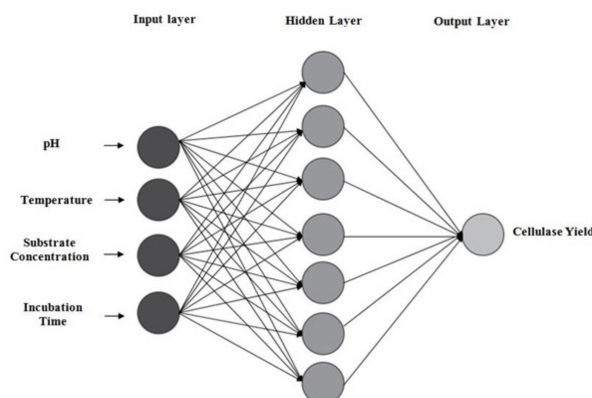

Figure 1. The topology of a neural network for the estimation of cellulase yield.

Different concentrations of substrate and range of cultural conditions were given as input and the cellulase yield was taken as the output variable. Normalized values were given as input to the program. In this study, the input layer consisted of 4 neurons (pH, temperature, substrate concentration, and incubation time), the hidden layer consisted of 7 neurons and the output layer consisted of one neuron (yield). The data is propagated in a feed-forward way from the input layer to the output layer. The neurons from the input layer to the hidden layer and then, hidden layer to the output layer were interconnected through synapses with an assigned weight. The data between two neurons is presented in the form of a variable parameter called weight. The data from the input layer is transferred to the hidden layer in the form of weight. The hidden layer sums up the input weights along with the biased input and the output is produced by an activation function. The back-propagation algorithm adjusts the weights in the three layers in such a way that the error is minimized. The network is fully trained with the back-propagation algorithm and the mean square errors were calculated from required and determined output values of each neuron in the output layer. In this experiment, the data set consists of 30 samples, out of which $3/4^{th}$ of the samples were used for the training process, and the remaining $1/4^{th}$ samples were used for the validation and testing process. The accuracy and validation of both RSM and ANN models are examined by statistical factors such as absolute average deviation (ADD), standard error prediction (SEP), root mean square error (RMSE), model predictive error (MPE), (R^2) correlation coefficient, bias (Bf), accuracy factor (AF) and chi-square (X^2) statistic factors were used. All the accuracy evaluating factors are executed using MATLAB 2009a.

RESULTS AND DISCUSSION

Isolation and characterization of cellulase producing isolates

Among the thirty-seven different colonies isolated from decomposing logs of textile industries Surat, India, an isolate vs15 was selected. It showed a cellulase activity of 1.21 IU/ml with a clear zone of 1cm. It showed a filamentous structure under the microscope (Figures 2A, 2B, and 2C). Phylogenetic analysis of the 16S rRNA gene revealed the isolate vs15 to be closely related to the species *Streptomyces durhamensis* as shown in Figure 3. The sequence of *Streptomyces durhamensis* vs15 was deposited in GenBank under accession number KT961129.

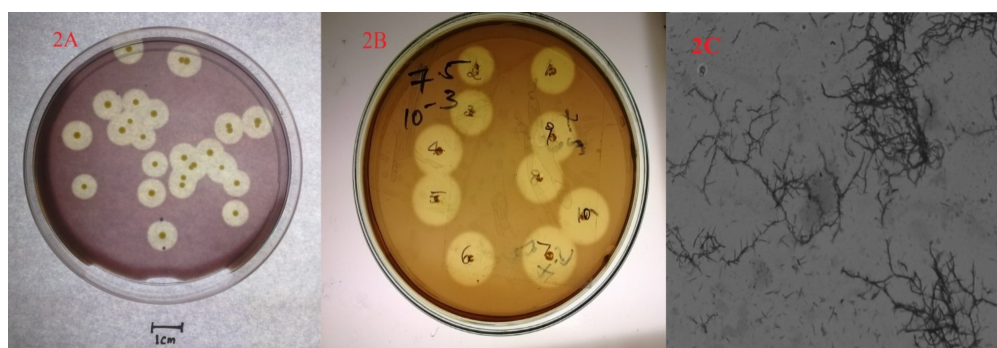

Figure 2. (A) Cellulase producing isolate *Streptomyces durhamensis* vs15 (Wild) (B) *Streptomyces durhamensis* GC23 (Mutant) with a zone of clearance detected by Gram's iodine staining (C) Microscopic view of *Streptomyces durhamensis* vs15

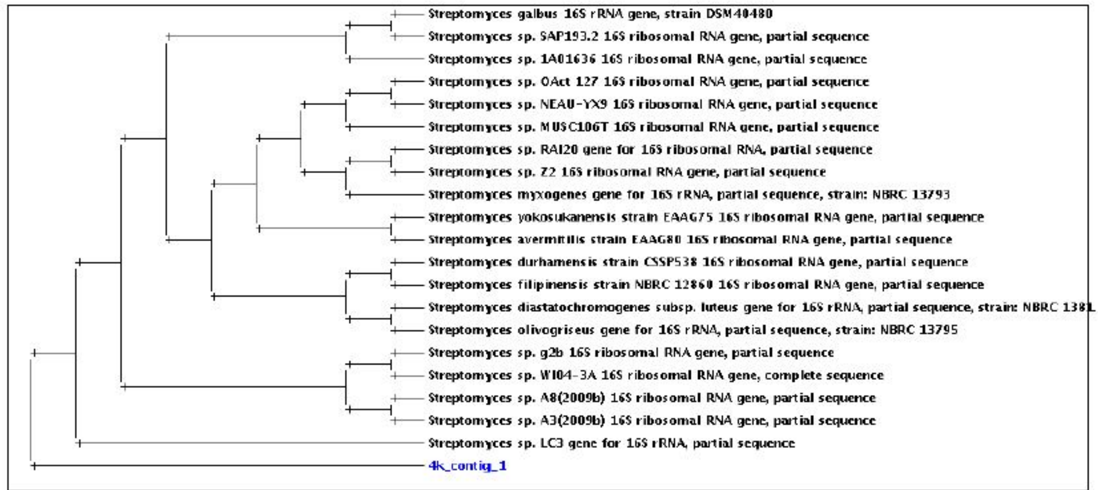

Figure 3. Phylogenetic tree derived from 16s rRNA sequence of *Streptomyces durhamensis* vs15, and sequences off related strains obtained from NCBI database.

Enhancement of cellulase production by Mutation

A total of 680 mutants were isolated using various mutagens (UV, EMS, and NTG) with different combinations. Mutants of the UV, EMS, and NTG were selected based on large halos around the colonies and faster growth. For this selection, the strains were twice subcultured, and reducing sugar was estimated by the DNS method. Among 680 mutants, GC23, obtained by the combination of UV and EMS mutagens and, having higher cellulase activity was selected. Mutants obtained by treatment (1, 2, 3 & 5) produced less cellulase when compared to the treatment 4 (UV+EMS). The cellulase activity of *Streptomyces durhamensis* GC23 was 2.25 IU/ml, enhanced by 1.86-fold over that of wild type *Streptomyces durhamensis* vs15 (1.21 IU/ml) as shown in (Fig 2B).

Various doses of the mutagen's EMS (0.62-1.48 mM), NTG (2-5μg), and UV (1-15 min) were tested to find out the optimum dose. The lethality rate of vs15 reached 100% when exposed to UV radiation for 10 min. By UV irradiation mutagenesis, 143 isolates were screened. Among these, GCU6 (obtained at 5 min UV exposure) revealed the highest cellulase production of 1.8I U/ml with a zone of 1cm. GCU6 had a 1.5 fold higher cellulase enzyme activity compared to that of the wild type (1.21 IU/ml).

Streptomyces durhamensis vs15 was subjected to EMS treatment for enhancing cellulase production. The lethality rate of vs15 reached 100% when exposed to 1.24 mM of EMS. After the EMS treatment, among the 257 mutants screened, isolate GCE29 (obtained at 0.931 mM of EMS concentration) showed the highest cellulase activity of 2.09 IU/ml with a zone of 1.4 cm.

Cells of *Streptomyces durhamensis* vs15 were subjected to NTG mutagenesis. The lethality rate of vs15 reached 100% when exposed to 4μg of NTG placed at the center of the plate. Among the 280 mutants isolated, GCN5 (obtained at 2.5 μg NTG) showed the highest cellulase activity of 1.91 IU/ml placed at the center of the plate with a zone of 1.4 cm. Mutant GCN5 had 1.58 fold more cellulase enzyme activity compared to that of wild type (1.21 IU/ml),

By the combination of 0.931 mM of EMS concentration with 5 min UV irradiation treatment, 74 mutants were isolated. Among these, the activity of GC23 was found enhanced to 2.25 IU/ml, which showed a clear zone of 1.6cm. No improved activity was observed with the combined treatment of 2.5 μg of NTG with UV irradiation.

Mutagenesis has been widely used as an efficient tool for strain improvement. Many studies have been conducted to improve the strain through mutagenesis. For example, *Aspergillus sp.*XTG-4s treated with γ, UV-irradiation, and NTG mutagens enhanced cellulase activity by 2-fold as compared to the wild type (VU; PHAM; KIM, 2009). In *F. oxysporum,* cellulase activity was enhanced 3-fold upon successive treatment with UV and NTG (KUHAD; KUMAR; SINGH, 1994; KUMAR; PARIKH, 2015) reported a 4.9-fold higher *β*-glucosidase activity by a mutant strain of *Aspergillus terreus* D34 obtained from EMS treatment compared to its parent strain. In our study, the activity of GC 23 was enhanced to 2.25 IU/ml, with a clear zone of 1.6 cm obtained with the combination of 0.931 mM of EMS with 5 min UV irradiation treatment.

Optimization of cellulase activity using central Response Surface Methodology (RSM)

The central composite experimental design was used to study the culture conditions influencing cellulase production. The environmental factors such as pH, temperature, and incubation time and substrate concentration were given as input variables and they were further randomized in thirty experiments. The design matrix was optimized with six central points, 16 cubic points, and eight axial points. The response such as cellulase yield were studied by applying multiple regression analyses (KUMAR; PARIKH, 2015). The results were given by overall second-order polynomial equations for cellulase production and cellulase activity (IU/ml) =+2.46+0.0194*A+0.0130* B+0.0539* C+0.0454* D-0.0013AB+0.0044 AC-0.0004 AD+0.0127 BC+5.938E-003* BD+0.0102 CD-0.0420 A^2-0.0176 B^2-0.0407 C^2-0.0297 D^2.

where Y1 is the cellulase yield and A, B, C, and D are the coded terms for the four test factors, i.e. pH, temperature, substrate concentration, and incubation time, respectively. The statistical implication of the model equation was calculated by F-test. It was further used for the analysis of variance (ANOVA), which indicated that the regression is significant at 99% (P< 0.0001) confidence level for both responses. P-values indicated the significance of each of the coefficient and its importance was to understand the pattern of the mutual interaction between each of the variables. In the case of enzyme yield response (Y1), the factors A, B, C, D, A2, B2, C2, and D2 were significant model terms. ANOVA table for cellulase yield exhibited F-values of 24.26 (Table 2). The greater F–value indicates that the model was significant at a high confidence range. The R^2 values of cellulase yield are 0.9577, indicating that the models can predict the responses and could explain 96% of the variability in the yield. The high value of Adjusted R^2 of 0.9182, further, suggests the accuracy of the model and the number of variations that can be explained by the model. The probability value (<0.01) indicates the significance of the model.

Table 2. ANOVA of central composite design (CCD) for cellulase yield.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.2297	14	0.0164	24.26	< 0.0001	Significant
A-pH	0.0096	1	0.0096	14.19	0.0019	
B-Temperature	0.0044	1	0.0044	6.47	0.0225	
C-substrate conc	0.0691	1	0.0691	102.19	< 0.0001	
D-Incubation	0.0482	1	0.0482	71.32	< 0.0001	
AB	0.0000	1	0.0000	0.0237	0.8798	
AC	0.0002	1	0.0002	0.3108	0.5854	
AD	2.500E-07	1	2.500E-07	0.0004	0.9849	
BC	0.0023	1	0.0023	3.34	0.0878	
BD	0.0005	1	0.0005	0.7485	0.4006	
CD	0.0019	1	0.0019	2.86	0.1113	
A^2	0.0485	1	0.0485	71.67	< 0.0001	
B^2	0.0084	1	0.0084	12.48	0.0030	
C^2	0.0454	1	0.0454	67.06	< 0.0001	
D^2	0.0241	1	0.0241	35.69	< 0.0001	
Residual	0.0101	15	0.0007			
Lack of Fit	0.0089	10	0.0009	3.55	0.0873	not significant
Pure Error	0.0013	5	0.0003			
Cor Total	0.2399	29				

In RSM studies, suitable concentrations of different variables, and the relation between them was predicted by generating 3D and 2D plots. The 3D and 2D plots were produced for the interactions cyclically between the two variables and further, the individual response is graphed to show the optimum value of the media component (FANG et al., 2012). The 2D contour plot is a two-dimensional

representation of the response across the input variables (NARAYANAN; RAMANA, 2012). The 3D surface plot is a projection of the contour 2D plot giving shape and color. Figure 4 exhibits the 3D plots showing the interaction between various media parameters in the case of cellulase yield.

In every plot, the two test variables were varied within the experiment range while the rest of the variables were set to zero. Figure 4A shows the effect of the interaction of temperature (40°C to 60°C) and pH (3.5 to 5.5) on cellulase production. The maximum cellulase yield of 2.5 IU/ml - was observed at 55°C and pH 5. The effect of temperature on enzyme activity increased gradually up to 50°C. However, beyond 50°C, there was a decline in the activity. The enzyme activity decreased from pH 3.5 to 5.

Figure 4B depicts effect of interaction of substrate concentration (0.5% to 2.5%) and pH (3.5 to 5.5) on cellulase activity. The maximum yield of cellulase was observed with an increase in substrate concentration from 1.5% to 2 % and pH from 4.0 – 5.0. Figure 4C represents the interactive effects of incubation time (16-48 h) and pH (3.5-5.5) on cellulase production. The maximum cellulase yield was observed at 40 h and pH 5. The yield showed a steady increase between 32 h to 40 h of incubation time.

Figure 4D portrays the interactive effect of temperature (40 °C to 60 °C) and substrate concentration (0.5% to 2.5%) on cellulase activity. The maximum cellulase yield was obtained at 55°C and with 2% (w/v) of substrate concentration. Figures 4E and 4F showed the interactive effect of incubation time with temperature and substrate concentrations. Cellulase activity increased with increasing temperature, substrate concentration, and incubation time up to 55°C, 2% (w/v), and 40h, respectively. Further increase in temperature, substrate concentration, and incubation led to a reduction in cellulase activity.

The main aim of the response surface model is to track efficiently for the optimum values of the variables such that the response is maximized. Each counter plot characterizes an infinitive number of combinations of the two independent variables while the other variable is maintained at the center point (MA et al., 2014).

Response surface methodology (RSM) is a statistical optimization and mathematical technique to increase the yield, which is influenced by various factors and these techniques, also defines the effect of variables individually as well as with combinations on the processes, without any cost. Various factors can affect cellulase production such as temperature, pH, and incubation period. Among them, the temperature is one of the effective variables for cellulase production. According to (DEEPAK et al., 2008), the maximum production of cellulase by *Bacillus subtilis* AS3 was observed at 39 ºC. Other reports state that optimal growth for cellulase production was at 30 °C by *Penicillium* sp. and *Enhydrobacter* sp. ACCA2 respectively (PRASANNA; RAMANJANEYULU; REDDY, 2016; PREMALATHA et al., 2015). Our results state that maximum cellulase production can also be observed at a high temperature of 55ºC and it is the optimum temperature for maximum cellulase production. Beyond this temperature, enzyme activity may have reduced due to protein denaturation. The pH plays an important role in many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane (LIANG et al., 2010).

Recent studies show that *Aspergillus niger* and *Trichoderma sp.* produced maximum enzyme activity at pH 6.5 (GAUTAM et al., 2011). In the present study, the optimum pH for maximum cellulase production is 5.0 which is slightly acidic when compared to the previous reports. In terms of the incubation period, maximum cellulases production was reported for *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 after 60 h incubation (ACHARYA; CHAUDHARY, 2012). The decrease in enzyme production observed beyond the optimized incubation period could be due to the depletion of nutrients and the production of toxic metabolites. In the present study, the optimum incubation period for maximum production of cellulase is 40 h.

Using response surface methodology, several reports stated that enhanced production of the enzyme can be achieved by optimizing the media components, culture conditions (temperature, pH, substrate concentration, and inoculum size), etc. Enhanced production of cellulase was obtained by optimizing the media components in *Cellulomonas fimi NCIM-5015* (ALI; MUTHUVELAYUDHAM; VIRUTHAGIRI, 2013). The cellulase production in *Bacillus s*p. increased by optimizing the culture conditions such as temperature, pH, and inoculum size (VASUDEO; LEW, 2011). Using RSM, cellulase production was enhanced by optimizing the culture conditions of *Bacillus subtilis* vs15 (EGA et al., 2016).

Another study reported that enhanced cellulase production in *Rhizopus oryzae* MTCC 9642 was obtained by optimizing the culture parameters like substrate concentration, cultivation temperature, and pH (KARMAKAR; RAY, 2010).

A recent study reported that *B. cereus* strain produced maximum cellulase by optimizing the media components through RSM (TABSSUM et al., 2018).

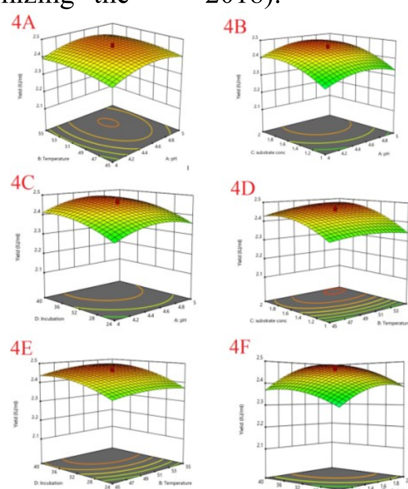

Figure 4. Three-Dimensional response surface plot for cellulase production showing the interactive effects of pH, temperature, incubation time and substrate concentration.
(A) pH and temperature while keeping substrate concentration and incubation time constant. (B) pH and substrate concentration while keeping temperature and incubation time constant. (C) pH and incubation time while keeping temperature and substrate concentration constant. (D) Temperature and substrate concentration while keeping Ph and incubation time constant. (E) Incubation time and temperature keeping Ph and substrate concentration constant. (F) Incubation time and substrate concentration while keeping temperature and pH constant.

Multilayer perceptron model and comparison of ANN and RSM models

In order to check the accuracy and validity of the RSM model, the ANN prediction model was proposed. Multilayer perceptron three-layered model consisted of 4 input neurons, 7 hidden neurons, and 1 output neuron. The four variables given as input to the model were pH, temperature, and substrate concentration, and incubation time. The output neuron is cellulase yield. The experimental output obtained from RSM was given as an input to the ANN model.

The number of hidden layers in the model was determined using the mean squares error (KIM; AO; AMOUZEGAR; RIEGER, 2013). Training of ANN was done by considering the numbers of hidden layers to be 1 and MSE values were noted. Thereafter, the MSE values were calculated by increasing the number of hidden layers to two and the process was continued up to 10 hidden layers. The number of hidden layers that showed the lowest MSE values was considered. Since the least MSE values were shown at 7 hidden layers, they were considered of optimized ANN.

The optimum levels of media variables for cellulase yield as shown in the experimental output were similar to that of the predicted values by RSM and ANN. The optimum levels were pH 5.0, the temperature at 55°C, substrate concentration at 2% (w/v), and an incubation time of 40 h. The experimental and predicted values for cellulase yield by RSM and ANN were 2.5 IU/ml and 2.53 IU/ml, respectively. In this study, strain improvement through mutagenesis of *Streptomyces durhamensis vs15* was achieved by increasing the cellulase production to 186% and was further optimized to 209.1% using RSM and ANN.

The experimental and predicted values obtained from the RSM and ANN models are shown in Table 3.

Table 3. Comparison of experimental with predicted values by CCD and ANN.

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1		
Std	Run	A: pH	B: Temperature	C: substrate conc.	D: Incubation	Yield IU/ml		
						Exp.	RSM	ANN
14	1	5	45	2	40	2.421	2.43	2.43
11	2	4	55	1	40	2.331	2.30	2.32

25	3	4.5	50	1.5	32	2.461	2.46	2.46
21	4	4.5	50	0.5	32	2.17	2.19	2.18
29	5	4.5	50	1.5	32	2.459	2.459	2.46
23	6	4.5	50	1.5	16	2.25	2.256	2.25
16	7	5	55	2	40	2.50	2.49	2.49
27	8	4.5	50	1.5	32	2.45	2.46	2.45
19	9	4.5	40	1.5	32	2.37	2.36	2.37
10	10	5	45	1	40	2.341	2.32	2.33
30	11	4.5	50	1.5	32	2.429	2.46	2.44
24	12	4.5	50	1.5	48	2.41	2.43	2.42
5	13	4	45	2	24	2.251	2.28	2.26
20	14	4.5	60	1.5	32	2.387	2.41	2.41
9	15	4	45	1	40	2.26	2.28	2.27
13	16	4	45	2	40	2.396	2.38	2.38
2	17	5	45	1	24	2.231	2.26	2.25
18	18	5.5	50	1.5	32	2.321	2.33	2.33
26	19	4.5	50	1.5	32	2.469	2.46	2.46
3	20	4	55	1	24	2.201	2.22	2.21
17	21	3.5	50	1.5	32	2.24	2.25	2.24
8	22	5	55	2	24	2.361	2.37	2.36
22	23	4.5	50	2.5	32	2.402	2.40	2.40
4	24	5	55	1	24	2.28	2.25	2.27
15	25	4	55	2	40	2.446	2.44	2.44
12	26	5	55	1	40	2.331	2.33	**2.33**
6	27	5	45	2	24	2.342	2.33	**2.34**
7	28	4	55	2	24	2.343	2.32	**2.33**
1	29	4	45	1	24	2.261	2.23	**2.23**
28	30	4.5	50	1.5	32	2.473	2.46	**2.47**

Bold represents the testing data of ANN analysis

To evaluate and predict the fitting and accuracy of the ANN and RSM model, error functions are calculated by equations which are shown in Table 4. The error values for RMSE (0.0199), MPE (0.7247), MAE (0.0170), ADD (0.7267) and SEP (0.8467) were calculated for RSM model. The values are higher when compared to those of ANN. RSME (0.0180), MPE (0.6445), MAE (0.0150), ADD (0.6456) and SEP (0.7644) represent error values for ANN. The error values of RSM and ANN are shown in (Table 4). The lesser error values for ANN indicated the higher optimizing ability of the model when compared to RSM.

Table 4. Statistical parameters for RSM and ANN Models.

S.No	Statistical parameters	RSM Predicted	ANN Predicted
1	**RMSE**= $\sqrt{\sum_{i=1}^{n}(y_{i,e}-y_{i,p})^2/n}$	0.0199	0.0180
2	**AAD**= $\{[\sum_{i=1}^{p}(\lvert y_{i,e}-y_{i,p}\rvert y_{i,e})]/n\}\times 100$	0.7267	0.6456
3	**MPE**= $\frac{100}{n}\sum_{i=1}^{n}\left\lvert\frac{y_{i,e}-y_{i,p}}{y_{i,p}}\right\rvert$	0.7247	0.6445

4	**MAE**$=\frac{i}{n}\sum_{i=1}^{n}\lvert y_{i,e-}y_{i,p}\rvert$	0.0170	0.0150
5	**SEP**$=\frac{RMSE}{Y_e}\times 100$	0.8467	0.7644
6	$\mathbf{R^2}=\frac{\sum_{i=1}^{n}(y_{i,p}-y_{i,e})}{\sum_{i=1}^{n}(y_{i,p}-y_{i,e})^2}$	0.9511	0.9601
7	$\mathbf{X^2}=\sum_{i=1}^{n}\frac{(y_{i,p}-y_{i,e})^2}{y_{i,p}}$	1.6715e-004	1.3506e-004
8	$\mathbf{A_f}=10(\sum_{i=1}^{n}\lvert\log(y_{i,p}/y_{i,e})\rvert/n)$	0.0726	0.0645
9	$\mathbf{B_f}=10(\sum_{i=1}^{n}\log(y_{i,p}/y_{i,e})\ /n)$	0.0217	0.0075

Basri et al. (2007) compared the synthesis of Lipozyme-using wax ester from oleyl alcohol and palm oil using response surface methodology and ANN and stated that both the models provided good quality predictions but ANN showed clear superiority over RSM, in both estimation capabilities and data fitting. Nelofer et al.,(2012) also compared the optimization of recombinant lipase production in *Escherichia coli* BL21 by RSM and ANN and stated that ANN prediction was superior to RSM. In our study, ANN showed superiority in optimizing the cellulase activity of *Streptomyces durhamensis vs15* (2.53 IU/ml) as compared to RSM-predicted levels (2.51 IU/ml) which are closer to the observed data, suggesting that ANN optimization is a better method than RSM for cellulase production.

CONCLUSION

The study proves that the improvement in cellulase production can be obtained by the combination of EMS, NTG, and UV mutagenesis. Also, further improvement can be obtained by optimization of growth conditions using Response Surface Methodology and Artificial Neural Network. This is a first report of cellulase production by *Streptomyces sp.*

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RESUMO: Trinta e sete colônias diferentes foram isoladas de toras em decomposição das indústrias têxteis. Dentre estes, uma bactéria do solo filamentosa termotolerante, Gram-positiva, Streptomyces durhamensis vs15, foi selecionada e rastreada quanto à produção de celulase. A cepa mostrou uma formação de zona clara na placa de ágar CMC após a coloração com iodo Gram. Streptomyces durhamensis vs15 foi ainda confirmado para a produção de celulase, estimando os açúcares redutores pelo método do ácido dinitrosalicílico (DNS). A atividade foi aprimorada por mutagênese sequencial usando três mutagênicos de irradiação ultravioleta (UV), N metil-N'-nitro-N-nitrosoguanidina (NTG) e metanossulfonato de etil (EMS). Após a mutagênese, a atividade celulase do GC23 (mutante) foi melhorada para 1,86 vezes em comparação com a cepa selvagem (vs15). As condições ideais para a produção de celulase pela cepa GC 23 foram avaliadas usando a Metodologia de Superfície de Resposta (RSM) e a Rede Neural Artificial (RNA). Os efeitos do pH, temperatura, duração da incubação e concentração de substrato na produção de celulase foram avaliados. As condições ideais para a produção da enzima celulase usando Carboximetilcelulose como substrato são 55 ° C de temperatura, pH de 5,0 e incubação por 40 h. A atividade da celulase do mutante Streptomyces durhamensis GC23 foi ainda otimizada para 2 vezes a atividade do tipo selvagem por RSM e RNA.

PALAVRAS-CHAVE: Streptomyces durhamensis. Mutagênese. Metodologia de superfície de resposta. Rede neural artificial. Carboximetilcelulose.

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