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CONVERSION OF WHEAT STRAW INTO FERMENTABLE SUGARS USING CARBOXYMETHYL CELLULASE FROM *TRICHODERMA VIRIDE* THROUGH BOX-BEHNKEN DESIGN AND ARTIFICIAL NEURAL NETWORK

Rubina Nelofer[1], *Muhammad Nadeem*[1], *Muhammad Irfan*[2*], *Quratulain Syed*[1], *Sara Nawaz*[3], *Arifa Tahir*[3]

Address(es): Dr. Muhammad Irfan,
[1]Pakistan Council of Scientific & Industrial Research Laboratories, Food & Biotechnology Research Center, Ferozpure road, 54600 Lahore, Pakistan.
[2]University of Sargodha, Faculty of Science, Department of Biotechnology, University Road, 40100 Sargodha, Pakistan.
[3]Lahore College for Women University, Biotechnology Department, Jail Road 54000 Lahore, Pakistan.

*Corresponding author: irfan.biotechnologist@gmail.com

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ABSTRACT

In this study, carboxymethyl cellulase was produced in submerged fermentation characterized and saccharification was optimized through Box-Behnken design. The optima pH and temperature of enzyme produced by *Trichoderma viride* were 5 and 50 oC, respectively. The crude enzyme had *Km* and *Vmax* values of 1.5143 µM and 0.9253µM/min, respectively, using carboxymethyl cellulose as substrate respectively. Three variables including pH (X1), incubation temperature (X2) and substrate concentration (X3) with three levels were used to optimize saccharification of wheat straw having 83% cellulose content using Box Behnken design and Artificial Neural Network (ANN). Results reveal that the proposed model was significant and quadratic effect of these parameters significantly affects the sugar production. Maximum sugar production (28.87 mg/ml) was predicted at RSM predicted levels of pH (6.6), incubation temperature (50 ºC) and (6.2%) substrate concentration, while the levels predicted for pH, temperature and substrate concentration were 5, 50 ^{o}C and 3.5 %, respectively, by ANN. The predicted sugar concentration at these levels was 30.72 mg/mL. The observed values at the predicted levels of RSM and ANN were 25.52 and 29.95 mg/mL respectively.

Keywords: Wheat straw, *Trichoderma viride*, Cellulase, Characterization, Response surface methodology, Artificial neural network

INTRODUCTION

Saccharification is a major step in production of bioethanol from lignocellulosic biomass. However, the enzyme cellulase is used as a tool that converts cellulose into fermentable sugars. Cellulase is a complex of three types of enzyme: exoglucanase, endoglucanase and β-glucosidase that have different modes of action in hydrolysis process **(Dawson and Boopathy 2008)**. The applications of cellulase range from food and paper to the textile industry as well as in production of biofuel from agro wastes. In nature, many microorganisms have inducible synthesis of cellulase that hydrolyzes cellulose to sugars that they used for their growth. Among these microorganisms, Trichoderma viride, Trichoderma *reesei* and *Aspergillus niger* are promising strains for cellulase production **(Ahamed and Vermette, 2008; Dhillon, *et al.*, 2011;Yasmin, *et al.*, 2013)**.
The cost of enzyme production is a main obstacle for its commercial production and it causes inexhaustible issues for research. Therefore, it needs to develop the economical production process for cellulases enzymes (**Gerdal, 2006**). Several methods have been reported but most feasible method for cellulase production is from lignocellulosic biomass. It can be produced from various biomasses like byproducts of agriculture (wheat straw, rice husk, corn cob and bagasse). Wheat straw is a byproduct of wheat that is produced in a large quantity annually (**Asghar *et al.*, 2015**). Some part of wheat straw is burnt in fields after harvesting and sometimes saved for use in other applications like animal feed, industry etc. It comprises of cellulose (34-34%) hemicellulose (20-24%) and lignin (10-19%) (**Kim and Holtzapple 2006**).
Saccharification of biomass is the main step that had major impact on production of biofuels and other beneficial molecules. Therefore optimum methods and efficient enzymes are unavoidable to lower the cost of biomolecules production process from biomass (**McIntosh and Vancov, 2011**). Optimization of enzymatic saccharification provides the maximum yield in the form of optimal levels of all effecting factors. Modern statistical methods including RSM and ANN have many advantages over conventional one parameter at a time technique and give a picture of the process closer to the actual optimal levels with the calculation of all other effects including the interactions and predictions beyond the levels of factors used in the experimental design (**Das *et al.*, 2015**).
Therefore, the present study was conducted for the optimization of wheat straw saccharification through indigenously produced cellulase enzyme using RSM and ANN. These methodologies well known for their capacities to optimize bioprocesses (**Baladhandayutham *et al.*, 2009; Wei, *et al.*, 2017; Uhoraningoga *et al.*, 2018**). It is a combination of mathematical and statistical techniques for designing experiment, models building, evaluating the effects obtained from factors and for finding desired responses from optimized parameters (**Pandiyan *et al.*, 2014**). This study was designed for production and characterization of crude CMCase and statistical optimization of saccharification process. The wheat straw saccharification optimization using ANN method was not found in literature according to our knowledge.

MATERIAL AND METHODS

Fungal Culture

The pure culture of Trichoderma viride was obtained from Microbiology Laboratory, Pakistan Council for Scientific and Industrial Research (PCSIR) Labs. Complex, Lahore, Pakistan and maintained on potato dextrose agar (PDA) slants. The culture was streaked on PDA slants and incubated at 35 ºC for 5 days up till the sporulation. The slants were then wrapped in polythene bags and kept in refrigerator at 4-10 ^{o}C for further use.

Pretreated Substrate

The pretreated wheat straw (**Nawaz *et al.*, 2018**) was used for saccharification which was obtained from FBRC, PCSIR Labs Lahore, Pakistan. The powder (2 mm mesh size) substrate (wheat straw) was sealed in polythene bags for further experiments.

Enzyme Production

Cellulase production was carried out in submerged fermentation using medium composition of (g/L); pretreated wheat straw 50, $(NH_4)_2SO_4$ 10, $CaCl_2$ 0.5, $MgSO_4.7H_2O$ 0.5 and KH_2PO_4 4.0 and pH was maintained at 5.0 with 1N

HCl/NaOH (**Nawaz *et al.*, 2018**). The medium was autoclaved at 121 °C for 15 min. After that, the medium was cooled and inoculated with 4 mL (v/v) of fungus spore suspension and incubated at 30 °C for 7 days. After the end of fermentation time, the fermented broth was filtered through muslin cloth followed by centrifugation at 8000xg for 10min at 4 °C. The clear supernatant obtained was used as crude cellulase enzyme source.

Enzyme characterization and kinetics

The crude enzyme obtained after fermentation was characterized. For this purpose different temperature (20-70°C) and pH (3-8) were used to get the optimal working levels. Different substrate concentrations ranging from 0-20 µmol/ mL were used to obtain the V_{max} and K_m values.

Optimization of wheat straw sccharification

The sccharification of wheat straw was conducted using indigenously produced cellulase enzyme. For optimization of saccharification, three parameters (pH, Temperature and wheat straw concentration) were optimized using Box Behnken design (BBD). Two methods of optimization including RSM and ANN were used for that purpose. A total 13 number of experiments were carried out with three parameters at three levels using BBD (Table 1 & 2). The obtained results were used for the prediction of experimental yield and optimal levels by RSM and ANN methods. Experiments were also conducted on the optimal predicted levels by the two methods. The selected ANN used 3 experiments for the selection of model, 7 experiments to train the network, and 3 experiments to test the model (Table 2). The topology of the network was consisted of three neuron layers, one input layer of three neurons, one hidden layer of 8 neurons, one output layer of 1 neuron (Figure 1). Saccharification in % was calculated as follows (**Irfan *et al.*, 2016**);

% saccharification = Reducing sugar (mg/ml)/Pretreated wheat straw used (mg/ml) × 100

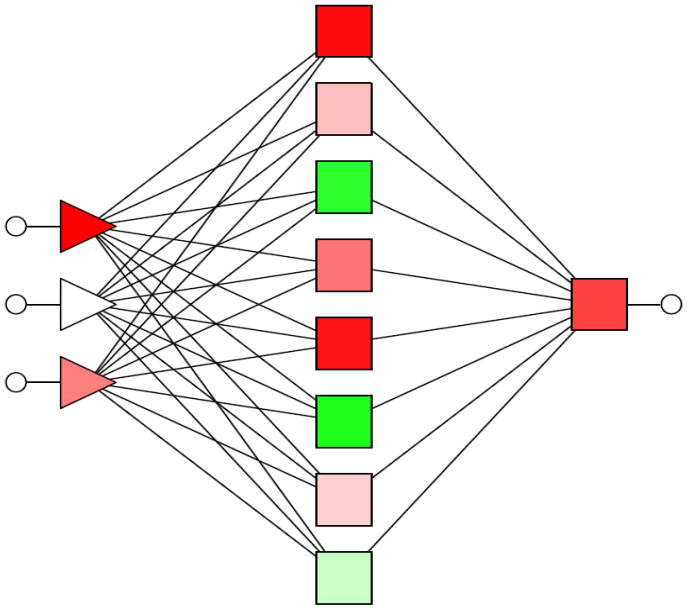

Figure 1 The Topology of the selected ANN showing the three layers structure.

Table 1 Variables and their coded levels used for Box Bhenken Design

Variables	Codes	Levels		
		-1	0	1
pH	X1	3	5	7
Temperature	X2	30	50	70
Substrate Conc. (%)	X3	1	3	5

Analytical Methods

Cellulase activity was determined with 1 % (w/v) of carboxymethyl cellulose (CMC) as substrate, in 50 mM Na-citrate buffer having pH 4.8 and incubating at 50°C for 30 min. The reducing sugars liberated during the reaction was estimated DNS method of **Miller (1959)**. One unit (IU) of endoglucanase was defined as the amount of enzyme that released 1 µmol of glucose equivalent per min under the assay conditions. Reducing sugars were measured by DNS method using glucose as standard.

Statistical analysis

Experiments were designed and analyzed statistically by STATISTICA software v.7. The variables like pH (X1), Temperature (X2) and substrate concentrations (X3) having three levels are mentioned in table 1 were used for optimization through Box Bhenken design using RSM and ANN methods.

RESULTS AND DISCUSSION

In this study endoglucanase (CMCase) enzyme was produced from *Trichoderma viride* in submerged fermentation using wheat straw as substrate. The crude endoglucanase enzyme was characterized and further utilized for hydrolysis of wheat straw for production of sugars through statistical optimization. The optima pH, temperature and kinetics of crude endoglucanase were determined.

Characterization of the endoglucanase enzyme

The enzyme activity was tested at various pH ranges from 3.0-8.0 and results (Fig. 2) showed that endoglucanase enzyme exhibited maximum activity at pH 5. Further change in pH beyond this resulted decline in enzyme activity. Results of this study was consistent with previous reports. Previously published literature showed that endoglucanase enzyme had optimum pH of 5 produced from *Trichoderma viride* (**Bhattacharya *et al.*, 2014**). Some strains of *Trichoderma* sp. produced endoglucanase enzyme having optimum pH of 3.0 (**Andrade *et al.*, 2011**). The optimum pH of 4.0 (**Yasmin *et al.*, 2013**) and 6.0 (**Taha *et al.*, 2014**) had been reported for endoglucanase from *Trichoderma viride*.

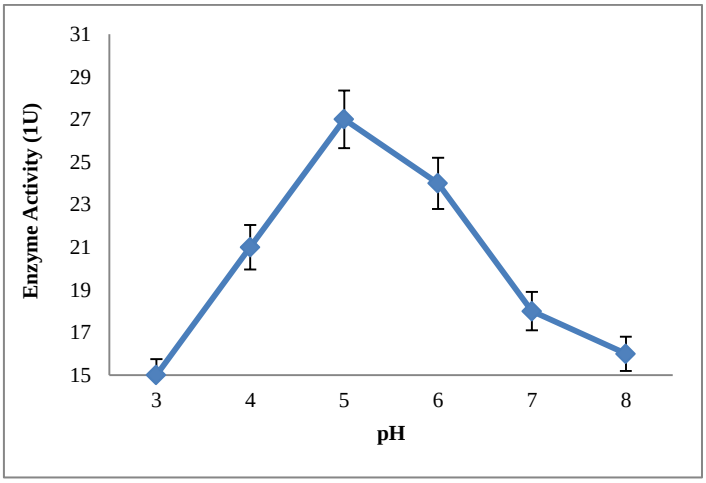

Figure 2 Effect of different pH on CMCase activity produced from *Trichoderma viridi* in submerged fermentation.

Endoglucanase activity was checked at different temperature ranging from 25-60 °C to find the optimum temperature of enzyme. Results (Fig. 3) revealed that enzyme activity was increased by increasing temperature from 25 °C to onward and peak activity was observed at 50 °C. Further increase in temperature led to decline in enzyme activity. Our findings were in line with previous literature that also showed the optimum temperature of 50 °C from *Trichoderma viride* (**Yasmin *et al.*, 2013; Bhattacharya *et al.*, 2014; Taha *et al.*, 2014**). **Iqbal *et al.*, (2011)** reported 55 °C optimum temperature of cellulase produced from *Trichoderma viride*. **Andarde *et al.*, (2011)** reported that cellulases produced from *Trichoderma* sp. IS-05 had optimum temperature of 60°C.

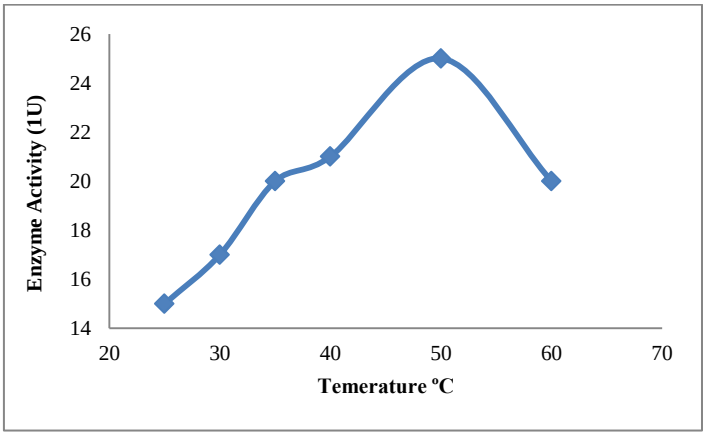

Figure 3 Effect of different incubation temperature on CMCase activity produced from *Trichoderma viridi* in submerged fermentation.

Kinetics of the crude endoglucanase was studied by carboxymethyl cellulose as a substrate. Vmax and Km value was found to be 1.5143 µM and 0.9253µM/min (Fig. 4). Cellulase produced from *Trichoderma longibrachiatum* exhibited Km and Vmax of 0.121 mg/ml and 0.421 µmol/min using carboxymethyl cellulose as substrate (**Pachauri *et al.*, 2017**). **Bhattacharya *et al.*, (2014)** reported Km and Vmax values 0.53mg/ml and 83.7ug/min for cellulase produced from Trichoderma viride respectively. The cellulase enzyme produced from *Trichoderma viride* exhibited Km and Vmax values of 68µM and 148U/ml respectively (**Iqbal *et al.*, 2011**). Another study conducted on cellulase produced from *Trichoderma viride* revealed the Km and Vmax of 2.5 x 10-5 g/l and 75 $g/lmin^{-1}$ mg^{-1} respectively.

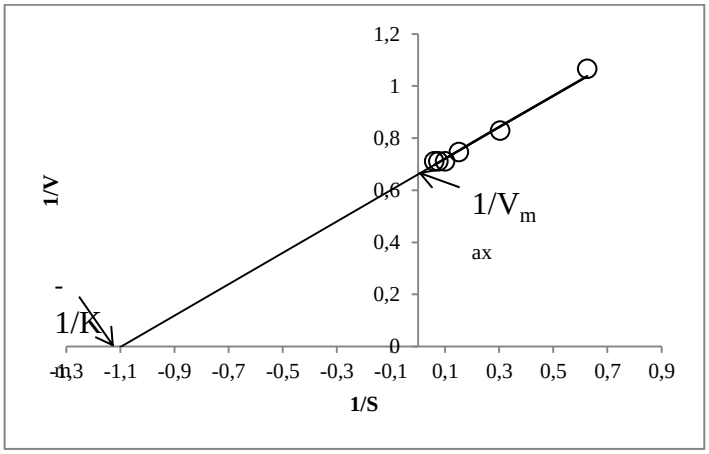

Figure 4 Lineweaver-Burke plot of rate of celluase reaction with different substrate concentrations.

Optimization of pretreated wheat straw saccharification

Response surface methodology (RSM) is a promising method that is valuable for screening and characterization of various parameters that have significant effects on operating specifications (**Thanapimmetha *et al.*, 2012**). The close values of observed and predicted response indicated the accuracy of the model (Table 2). Maximum sugar (24.6 mg/ml) was released at substrate concentration of 1.0 %, pH 5.0 and temperature of 50 ^{o}C. The response obtained was calculated through polynomial regression as given below.

$$Y\ (\text{Sugar mg/ml}) = -121.244 + 19.656\ X1 + 3.18\ X2 + 55.350X3 + (-1.791)\ X1^2 + (-0.029)\ X2^2 + (-19.350)\ X3^2 + (-0.019)\ X1X2 + (-0.100)\ X1X3 + (-0.243)X2X3$$

Where y is the measured response and X1, X2, X3 are coded independent variables.

The analysis of variance (ANOVA) was performed to check the effectiveness of the variables. The model was significant having F-value of 22.013 corresponding P-value of 0.013 (Table 3). The coefficient of determination (R^2) of the proposed model was 0.992. The linear and square effects were also found very significant affecting sugar production.

The optimal levels predicted by RSM were pH 6.6, substrate concentration of 6.6 and temperature of 50 ^{o}C with predicted response 28.87 mg/L. This response was confirmed through repeated experiment which was close to the prediction (25.52 mg, 37.88 % sccharification). The interaction of variables could be understood by plotting response surface plots. These response surface plots represent the three dimensional view of variable affecting sugar production. The interaction effect of temperature and substrate concentration is significant (Table 3) and it has appositive effect on sugar yield (Fig. 5). The most important objective of Response surface methodology (RSM) is to find out the optimum conditions within the operating specification (**Karmakar and Ray 2011**).

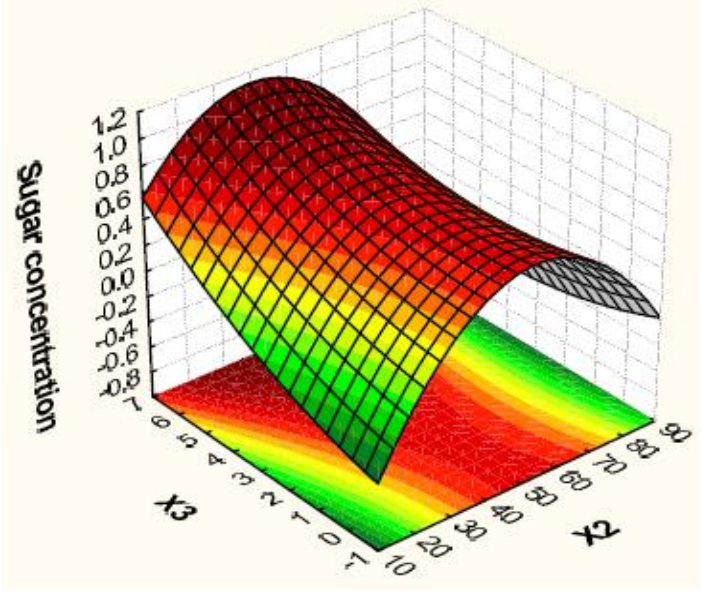

Figure 5 Response surface graph representing the interaction effect of ℃ (X2) and substrate concentration % (X3) on sugar concentration (mg/mL) calculated by RSM.

RSM method is already being used to optimize alcoholic fermentation (**Cristiane *et al.*, 2011**). The pervious study was reported that 90.24% saccharification was achieved from sugar cane tops by Box Bhenken design for the optimization of biomass loading (10%), enzyme loading (100 FPU/g of cellulase), surfactant concentration 0.04% (w/w) 72 h incubation time as reported by **Maurya *et al.*, (2013)**. **Irfan *et al.*, (2016)** reported 40.15% saccharification using 2% pretreated wheat straw. The earlier study was described RSM parameters where maximum of 3.36g/L ethanol production was achieved under optimum pH of 5.6, incubation temperature of 32ºC and fermentation time 110 h (**Chen *et al.*, 2010; Dhillon 2011**).

The Selected ANN on the other hand showed correlation coefficient very close to 1 for the whole model and for the selection, training and test experiments (Table 4), representing the prediction accuracy of the selected ANN. A total number of 50 networks were tested for the selection. The optimum levels predicted by ANN were 5, 50 ℃ and 3.5 % for pH, temperature and substrate respectively with predicted sugar yield of 30.73 mg/mL. The observed yield on those levels was 29.95 mg/mL (85.5% saccharification). The sensitivity test of ANN represents strength of factor's effect on the sugar yield. The maximum effect is of temperature (X2), the next effect is from substrate and the least effect from pH. It was found from the studies that ANN performed better for the wheat straw saccharification optimization in prediction of optimal levels and the sugar yield on those levels. However, the experimental yields of Box-Behnken design are closer to the predicted yields by RSM. The interaction effects are also quantified by RSM as compared to ANN (**Nelofer *et al.*, 2012**). The interaction effect of temperature and substrate concentration on sugar yield was represented in Figure 6 calculated by ANN.

In most of the cases in optimization of bioprocesses ANN performed better than the RSM (**Zafar *et al.*, 2012; Nelofer *et al.*, 2012; Siddique *et al.*, 2014; Titah *et al.*, 2018**). In the present studies ANN gave the prediction of optimal levels close to the actual with 85.5% saccharification of wheat straw. No studies were found on the saccharification optimization of wheat straw using ANN method. However maximum saccharifcation of wheat straw obtained by using RSM or other methods to optimize was lower in most of the cases than that obtained in the present study (**Hatakka, 1983; Saha *et al.*, 2006; Li *et al.*, 2009**).

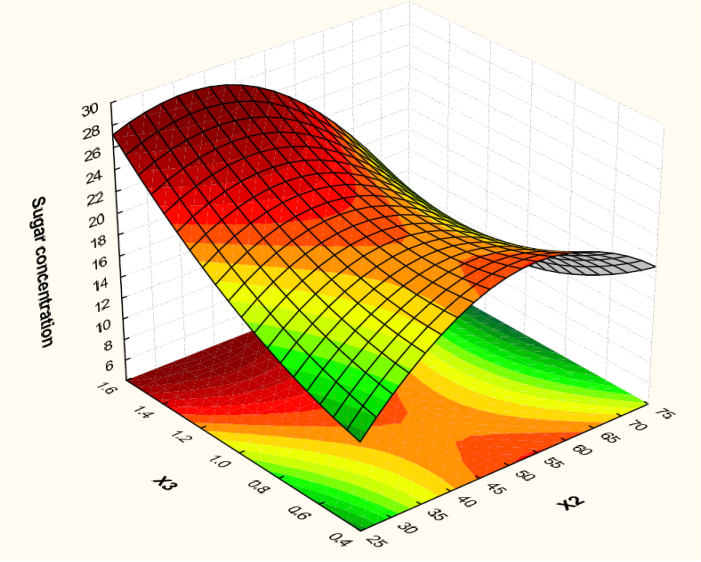

Figure 6 Response surface graph representing the interaction effect of temperature °C (X2) and substrate concentration % (X3) on sugar concentration (mg/mL) calculated by ANN.

Table 2 Box Behnken Design for Optimization of Saccharification by using RSM and ANN

Exp. No	pH (X_1)	Temp (X_2) (^oC)	Substrate Conc. (X_3)	Sugar (mg/ml)		
				Observed	Predicted by RSM	Predicted by ANN
1	*7*	*30*	*3*	*21.6*	*20.93750*	*23.25541*
2	7	70	3	15.0	15.51250	14.81959
3	*7*	*50*	*1*	*23.3*	*23.11250*	*23.20147*
4	7	50	5	26.6	26.93750	26.69827
5	3	30	3	17.1	16.58750	17.11524
6	3	70	3	13.5	14.16250	13.59531
7	3	50	1	20.4	20.06250	20.29684
8	3	50	5	24.1	24.28750	23.98887
9	*5*	*30*	*1*	*15.8*	*16.65000*	*16.74472*
10	5	70	1	17.9	17.57500	17.93143
11	5	30	5	25.2	25.52500	26.89628
12	5	70	5	17.6	16.75000	15.20147
13	5	50	3	24.6	24.60000	21.67446

*The experiments used for the selection of network are represented by *italic*, that used for training are represented by **bold** and the experiments used for testing the ANN are in normal font.

Table 3 ANOVA for sugar production using RSM

Source	Sum of Square	Degree of Freedom	Mean Square	F- Value	P- Value
Model	20.64271	1	20.64271	18.44472	0.023216
X_1	10.24111	1	10.24111	9.15066	0.056534
X_1^2	6.31750	1	6.31750	5.64483	0.097981
X_2	92.23505	1	92.23505	82.41404	0.002824
X_2^2	86.10036	1	86.10036	76.93256	0.003121
X_3	3.14201	1	3.14201	2.80746	0.192420
X_3^2	1.00321	1	1.00321	0.89639	0.413599
X_1X_2	2.25000	1	2.25000	2.01042	0.251243
X_1X_3	0.04000	1	0.04000	0.03574	0.862119
X_2X_3	23.52250	1	23.52250	21.01787	0.019489

Table 4 Statistical values calculated by ANN for the selection, training, testing and overall experiments.

Parameters	Training	Selection	Test	Overall
Data Mean	19.22857	20.23333	22.46667	20.20769
Data S.D.	4.41482	3.21075	3.44996	4.16108
Error Mean	-0.02206	0.83386	-1.20926	-0.09851
Error S.D.	0.10148	0.72032	2.06577	1.26433
Abs E. Mean	0.09071	0.89955	2.34012	0.79646
S.D. Ratio	0.02299	0.22435	0.59878	0.30385
Correlation	0.99974	0.97478	0.92459	0.95683

Table 5 Rank and ratio values calculated by ANN for the four parameters used in the experiments.

Parameters	X1	X2	X3
Ratio	1.346659	3.006365	2.310417
Rank	3.000000	1.000000	2.000000

CONCLUSION

The results of this study concluded that cellulase enzyme produced from *Trichoderma viride* actively work at pH 5.0 and temperature of 50ºC. The produced cellulase enzyme effectively hydrolyzed the wheat straw to liberate sugars which could be further used in fermentation process for the production of valuable products such as ethanol. ANN was found to be a better method for the prediction of optimal levels of three factors affecting the sugar yield as compared to RSM.

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