# Integration of Artificial Neural Network Modeling and Genetic Algorithm Approach for Enrichment of Laccase Production in Solid State Fermentation by *Pleurotus ostreatus*

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Black gram husk was used as a solid substrate for laccase production by *Pleurotus ostreatus*, and various fermentation conditions were optimized based on an artificial intelligence method. A total of six parameters, *i.e.*, temperature, inoculum concentration, moisture content, $CuSO_4$, glucose, and peptone concentrations, were optimized. A total of 50 experiments were conducted, and the obtained data were modeled by a hybrid of artificial neural network (ANN) and genetic algorithm (GA) approaches. ANN was employed to model the experimental data, and the predicted values were further optimized by GA. Employment of ANN–GA hybrid methodology resulted in a significant improvement, as approximately two-fold laccase production (4244 U/gds) was achieved.

*Keywords: Laccase; Artificial intelligence; Neural networks; Lignocellulolytic enzyme; Genetic algorithm; Optimization*

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## INTRODUCTION

Laccases (EC 1.10.3.2, p-diphenol: dioxygenoxidoreductases) are oxidoreductase enzymes that catalyze the oxidation of phenolic compounds by molecular oxygen (Neifar *et al.* 2011; Riva 2006). These are multi-copper-containing enzymes that catalyze the oxidation of a wide range of substrates by a radically catalyzed reaction mechanism with the concomitant reduction of oxygen to water in four electron transfer processes (Neifar *et al.* 2011). With this mechanism of action, laccases can detoxify various oncogenic substances, harmful pollutants, and synthetic dyes, which are effluents generated by the various pulp, paper, and textile industries. They have the ability to delignify wood pulp, which is a beneficial effect for the paper industry. These enzymes are also used in the food industry and for soil bioremediation, nanobiotechnology, various biosensors, synthetic chemistry, microbial fuel cells, and cosmetics (Bourbonnais *et al*. 1997; Kantelinen *et al.* 1989; Mishra and Kumar 2007; Srebotnik and Hammel 2000).

Reported sources of these enzymes include many microorganisms such as fungi, bacteria, yeast, marine algae, protozoans, and insects (Polizeli *et al.* 2005). Among these, fungi are a major group of microbes that are able to produce laccase in high amounts (Vivekanand *et al*. 2011). Laccases from white-rot fungi such as *Trametes versicolor*, *Coriolus versicolor*, *Phanerochaete chrysosporium*, and *Pleurotus* sp. (Landolo *et al.*

2011; Mishra and Kumar 2007; 2009; Tisma *et al.* 2012; Vivekanand *et al*. 2011) have been well studied in production as well as their industrial applications.

Production of industrial enzymes in a cost-effective manner is a prerequisite for their use in industrial processes. In the last decade, solid-state fermentation (SSF) has been shown to be an economically viable method for the production of various industrial enzymes (Hymavathi *et al*. 2009; Laxmi *et al.* 2008; Mahalaxmi *et al.* 2009; 2010; Sathish and Prakasham 2010). Various researchers have studied different solid substrates for the production of laccase, such as horticultural waste, tomato waste, and banana waste, and it has been reported that SSF is the best approach for economically viable production of lignolytic enzymes (Iandolo *et al.* 2011; Xin and Geng 2011). The production levels of these strains are low; thus further advances in the effective production of lignolytic enzymes on an industrial scale requires the isolation of high-yielding strains that are economically viable and readily available.

Optimization of the process and nutrient parameters is one of the best methods used to increase enzymes and metabolites production. Numerous optimization methods such as the conventional one-at-a-time method (Laxmi *et al.* 2008), response surface methodology (RSM) (Hymavathi *et al.* 2009), simplex method (Sathish *et al.* 2008), and orthogonal arrays (Mahalaxmi *et al.* 2009; 2010) are usually used for the optimization of media. The one-at-a-time method is laborious and time consuming, and often interaction effects are overlooked. Even though statistical methods have proven to be better methods for optimization than the one-at-a-time method, they have some limitations. In these methods, the number of parameters and levels are limited. The level of Taguchi or orthogonal array design is also limited by this factor (Fang *et al.* 2003). To overcome these problems, artificial intelligence based optimizations such as artificial neural networks (ANN) and genetic algorithms (GA) can be considered. Effective utilization of ANN and GA to enhance enzyme production in SSF by optimizing culture media has been reported (Sathish and Prakasham 2010). Instead, studies regarding the optimization of SSF for the production of laccase are very limited in scientific literature (Tisma *et al.* 2012). In the present study, various process and nutrient parameters that influence the laccase secretion in SSF were optimized based on a hybrid ANN-GA approach.

## EXPERIMENTAL

### Microorganism

An isolated, hyper laccase-producing white-rot fungus, *Pleurotus ostreatus* PVCRSP-7, was used in the present study. Its secretion of lignolytic enzymes was further improved during the optimization studies in SSF. The fungi was maintained on potato dextrose agar (PDA) plates and stored at 4 °C.

### Inoculum Preparation

An inoculum of *P. ostreatus* PVCRSP-7 grown on wheat grains was prepared according to Kumar and Chandra (1988). First, 200 g of wheat grains was placed in a 1000-mL Erlenmeyer conical flask, followed by the addition of two volumes of distilled water, and the solution was then boiled for 30 min. Boiled wheat grains were supplemented with 0.2% calcium carbonate and 1.2% calcium sulphate and sterilized at 15 lbs of pressure for 2 h. After sterilization, grains were cooled to room temperature and were inoculated with mycelial plugs grown on PDA agar plates and incubated at 25 °C.

Grains were ready for inoculation after 15 days of incubation. These mycelial cultivated wheat grains were used as the initial inoculum for the SSF studies.

## Solid State Fermentation Experiments

Three grams of *Vigna mungo* (black gram, local name) husk was taken in a 250-mL Erlenmeyer flasks and was moisturized with a salt solution (2.0% $KH_2PO_4$, 0.5 % $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2$, 0.5% KCl, and 1.0% urea) to set the desired moisture level. The media were then sterilized at 121 °C for 1 h to provide proper cooking of the substrate and to increase susceptibility to microbial attack. The media were cooled to room temperature after autoclaving and inoculated with 3 g of the inoculum of *P. ostreatus* prepared as stated above. The inoculated flasks were incubated in static conditions at 25 °C in an incubator. After 10 days of incubation, 0.15 mg/g substrate 2,5-xylidine inducer was added. The contents were mixed thoroughly four times daily during the fermentation period by gently hitting the flask bottom on the palm of the hand. All experiments were conducted in triplicate, and the results presented are mean values.

## Extraction of Laccase

After the given period of incubation, the fermented substrate cultures (3 g) were subjected to extraction of the enzyme. The laccase enzyme was extracted with chilled phosphate buffer (50 mM, pH 6.0) by a simple contact method as per Sathish *et al*. (2008).

The solid substrate was mixed with 25 mL of chilled buffer solution and kept for mixing in a rotary shaker (100 rpm) at 25 °C for 1 h. The homogenate was filtered through nylon cloth (200-mesh size), the procedure was repeated, and the filtrates were pooled. The pooled 50 mL of filtrate was centrifuged at 10,000 x *g* at 4 °C for 15 min. The clear supernatant was used for estimating the laccase activity.

## Estimation of Laccase Activity

Laccase activity was measured using the ABTS oxidation procedure according to Prasad (2005). Enzyme activity was expressed as units per g of dry substrate (U/gds) and was defined as the amount of enzyme producing 1 µM of product per min per g of substrate extracted.

## Optimization by Hybrid ANN-GA Modelling

*Data sets*

In the present study, the most promising factors that influence the laccase production were optimized using neural networks and genetic algorithms. In preliminary studies, temperature, moisture content, $CuSO_4$, size of inoculum, and glucose and peptone concentrations were found to be most predominant parameters influencing laccase production by *Pleurotus ostreatus*. These parameters were further optimized to increase the highest yield of laccase.

The experimental data used for ANN design are presented in Table 1. A central composite design with 50 experiments was employed. The data were divided into two sets: 40 runs of the data set were used for training the network, and 10 runs of the data set were used as testing data. The training data were used to compute the network parameters. The testing data were used to ensure robustness of the network parameters.

*Neural network modeling*

A feed-forward neural network, which uses an error back propagation-learning algorithm (BPNN), was constructed for modelling the laccase production. The network consists of three layers, *i.e.*, the input, hidden, and output layers. All three layers were connected to the subsequent layers; the connections are called weights. The weights played a vital role in the optimization of the data. Experimental conditions were chosen as inputs for the network, and the output was laccase activity. The number of neurons in the hidden layer was optimized based on a trial and error method (examined from 3 to 18). All of the data were normalized from -1 to +1. Scaled data passed through the input layer, were propagated from the input layer to the hidden layer, and finally passed to the output layer of the network. Every node in the input and the hidden layers is connected to the nodes in the subsequent layer. Each neuron in the hidden and output layers act as a summing junction that combines and modifies the inputs from the previous layer using the following equation,

$$Y_i = \sum_{j=1}^{n} x_i w_{ij} + b_j \quad (1)$$

where $Y_i$ is the net input to node $j$ in the hidden and output layers, $X_i$ is the output of the previous layer, $W_{ij}$ is the weights between the $i^{th}$ and $j^{th}$ node, $n$ is the number of neurons, and $b_j$ is the bias associated with node $j$.

The Sigmoid transfer function was used for the hidden layer, and the linear transfer function was used for the output layer to avoid error between observed and predicted values. During this process, the Levenberg-Marquardt algorithm was used for training the network. Initially, weight and bias values were taken randomly. However, in subsequent training steps, the weights and biases, in the hidden and output layers, were adjusted in accordance with a convergence criterion to obtain similarity in training and testing experimental titer values (Sathish and Prakasham 2010).

*Evaluation of ANN predictability*

To evaluate the ANN output error, the coefficient of determination, $R^2$, was used, which describes the extent of variance in the modeled variables. The error was calculated based on difference between the experimental and predicted values. A popular measure such as mean squared error (MSE) or root mean squared error (RMSE), mean absolute error (MAE), and mean absolute percentage error (MAPE) were used to evaluate the ANN simulated data,

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(y_p - y_e)^2 \quad (2)$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_p - y_e)^2} \quad (3)$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}\left|y_p - y_e\right| \quad (4)$$

$$MAPE = \frac{1}{n}\sum_{i=1}^{n}\frac{\left|y_p - y_e\right|}{y_e} \quad (5)$$

where $n$ is the number of experiments, $y_p$ is the ANN predicted value, and $y_e$ is the experimental value.

### GA Optimization

A genetic algorithm was used to search in different subspaces and to locate the global maximum on the objective function surface. The different parameters of GA, such as chromosome length ($L_{chr}$) as 36, population size ($N_{pop}$) as 36, cross over probability ($C_p$) as 0.7, and mutation probability ($P_{mut}$) as 0.01, were taken. Optimum conditions were selected after evaluation of GA for 300 generations ($Ng_{max}$ = 300) to achieve fine-tuned fermentation conditions in the given range of input parameters. Neural networks and genetic algorithm toolboxes of MATLAB 7.0 (Mathworks, USA) were used in modelling studies.

## RESULTS AND DISCUSSION

Agro-industrial residues are generally considered the best substrates for SSF processes, and production of enzymes using these materials as a substrate is no exception to that. A number of such substrates have been employed for the cultivation of micro-organisms to produce a host of enzymes, metabolites, and antibiotics (Hymavathi *et al*. 2009; Laxmi *et al*. 2008; Mahalaxmi *et al*. 2009; 2010; Sathish *et al*. 2008). In the present study, an isolated *P. ostreatus* PVCRSP-7 was employed for laccase production in SSF using black gram husk as a substrate. In preliminary studies it was noticed that (data not shown) lignolytic enzyme secretion by *P. ostreatus* is influenced by various environmental conditions and supplemented nutrients. Optimization of these parameters is essential for further improvement of laccase production by isolated *P. ostreatus* PVCRSP-7. A feed forward neural network coupled to genetic algorithm was employed to optimize the selected parameters in order to achieve higher amounts of enzyme.

Table 1 depicts the experimental design along with the observed lignolytic enzyme production by *P. ostreatus*. The enzyme production varied from 1266 to 3873 U/gds based on selected conditions. Observed minimum and maximum enzyme production indicates that selected parameters have a remarkable influence on laccase production. The data was further modeled with ANN, and conditions were optimized using the GA. For construction of ANN, the selected six variables, *i.e.*, incubation temperature, moisture content, $CuSO_4$ concentration, size of inoculum, and glucose and peptone concentrations, were chosen as input neurons in the input layer. Correspondingly, the laccase production was set as an output neuron in the output layer.

The number of neurons in the hidden layer was chosen by a trial and error method, varying the neurons from 3 to 18. A cross-validation criterion was used to fix the optimal number of neurons in the hidden layer. In all the structures studied, the number of epochs was fixed at 1000.

It was noticed that the hidden layer with eight neurons in the network resulted in the best correlation between the observed and predicted values, as well as the lowest MAPE and RMSE values. Further, the neural network was fixed with six, eight, and one neurons in the input, hidden, and output layers, respectively. Figure 1 depicts the constructed neural network topology.

**Table 1.** Experimental Design along with the Observed and ANN-predicted Laccase Activity Produced in SSF by Isolated *P. ostreatus* PVCRSP-7

| Run No | Temp (°C) | Moisture content (w/w) | $CuSO_4$ (mg) | Inoculum level (g) | Glucose (g) | Peptone (g) | Laccase activity (U/gds) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Obs[1] | Pred[2] | Res[3] |
| 1 | 24.00 | 50.00 | 0.4 | 2.50 | 0.05 | 0.05 | 2500 | 2500.56 | -0.56 |
| **2** | **24.00** | **50.00** | **0.4** | **2.50** | **0.25** | **0.25** | **2370** | **2370.29** | **-0.29** |
| 3 | 24.00 | 50.00 | 0.4 | 3.50 | 0.05 | 0.25 | 2654 | 2656.16 | -2.16 |
| 4 | 24.00 | 50.00 | 0.4 | 3.50 | 0.25 | 0.05 | 2886 | 2886.43 | -0.43 |
| **5** | **24.00** | **50.00** | **0.6** | **2.50** | **0.05** | **0.25** | **2855** | **2878.06** | **-23.06** |
| 6 | 24.00 | 50.00 | 0.6 | 2.50 | 0.25 | 0.05 | 2546 | 2545.51 | 0.49 |
| 7 | 24.00 | 50.00 | 0.6 | 3.50 | 0.05 | 0.05 | 3133 | 3132.2 | 0.80 |
| 8 | 24.00 | 50.00 | 0.6 | 3.50 | 0.25 | 0.25 | 3565 | 3495.58 | 69.42 |
| 9 | 24.00 | 70.00 | 0.4 | 2.50 | 0.05 | 0.25 | 2963 | 2961.94 | 1.06 |
| **10** | **24.00** | **70.00** | **0.4** | **2.50** | **0.25** | **0.05** | **1728** | **1727.78** | **0.22** |
| 11 | 24.00 | 70.00 | 0.4 | 3.50 | 0.05 | 0.05 | 3056 | 3056.98 | -0.98 |
| 12 | 24.00 | 70.00 | 0.4 | 3.50 | 0.25 | 0.25 | 3256 | 3254.82 | 1.18 |
| 13 | 24.00 | 70.00 | 0.6 | 2.50 | 0.05 | 0.05 | 2238 | 2237.88 | 0.12 |
| 14 | 24.00 | 70.00 | 0.6 | 2.50 | 0.25 | 0.25 | 3225 | 3224.62 | 0.38 |
| 15 | 24.00 | 70.00 | 0.6 | 3.50 | 0.05 | 0.25 | 3272 | 3271.93 | 0.07 |
| **16** | **24.00** | **70.00** | **0.6** | **3.50** | **0.25** | **0.05** | **2315** | **2316.57** | **-1.57** |
| 17 | 26.00 | 50.00 | 0.4 | 2.50 | 0.05 | 0.25 | 2500 | 2500.1 | -0.10 |
| 18 | 26.00 | 50.00 | 0.4 | 2.50 | 0.25 | 0.05 | 2778 | 2770.38 | 7.62 |
| 19 | 26.00 | 50.00 | 0.4 | 3.50 | 0.05 | 0.05 | 3210 | 3209.32 | 0.68 |
| 20 | 26.00 | 50.00 | 0.4 | 3.50 | 0.25 | 0.25 | 2917 | 2916.33 | 0.67 |
| 21 | 26.00 | 50.00 | 0.6 | 2.50 | 0.05 | 0.05 | 2485 | 2458.87 | 26.13 |
| 22 | 26.00 | 50.00 | 0.6 | 2.50 | 0.25 | 0.25 | 2978 | 2978.24 | -0.24 |
| **23** | **26.00** | **50.00** | **0.6** | **3.50** | **0.05** | **0.25** | **2114** | **2114.86** | **-0.86** |
| 24 | 26.00 | 50.00 | 0.6 | 3.50 | 0.25 | 0.05 | 2670 | 2678.37 | -8.37 |
| 25 | 26.00 | 70.00 | 0.4 | 2.50 | 0.05 | 0.05 | 2300 | 2299.65 | 0.35 |
| 26 | 26.00 | 70.00 | 0.4 | 2.50 | 0.25 | 0.25 | 2562 | 2746.32 | -184.32 |
| 27 | 26.00 | 70.00 | 0.4 | 3.50 | 0.05 | 0.25 | 2438 | 2437.84 | 0.16 |
| 28 | 26.00 | 70.00 | 0.4 | 3.50 | 0.25 | 0.05 | 2068 | 2067.03 | 0.97 |
| 29 | 26.00 | 70.00 | 0.6 | 2.50 | 0.05 | 0.25 | 2037 | 2037.26 | -0.26 |
| **30** | **26.00** | **70.00** | **0.6** | **2.50** | **0.25** | **0.05** | **1751** | **1769.00** | **-18.00** |
| 31 | 26.00 | 70.00 | 0.6 | 3.50 | 0.05 | 0.05 | 1266 | 1265.89 | 0.11 |
| 32 | 26.00 | 70.00 | 0.6 | 3.50 | 0.25 | 0.25 | 2130 | 2118.83 | 11.17 |
| **33** | **23.00** | **60.00** | **0.5** | **3.00** | **0.15** | **0.15** | **3364** | **3395.41** | **-31.41** |
| 34 | 27.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 2932 | 2746.32 | 185.68 |
| 35 | 25.00 | 40.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3411 | 3411.10 | -0.10 |
| 36 | 25.00 | 80.00 | 0.5 | 3.00 | 0.15 | 0.15 | 2639 | 2639.96 | -0.96 |
| 37 | 25.00 | 60.00 | 0.3 | 3.00 | 0.15 | 0.15 | 3256 | 3255.97 | 0.03 |
| **38** | **25.00** | **60.00** | **0.7** | **3.00** | **0.15** | **0.15** | **2978** | **2960.34** | **17.66** |
| 39 | 25.00 | 60.00 | 0.5 | 2.00 | 0.15 | 0.15 | 2994 | 3016.72 | -22.72 |
| **40** | **25.00** | **60.00** | **0.5** | **4.00** | **0.15** | **0.15** | **3426** | **3419.63** | **6.37** |
| 41 | 25.00 | 60.00 | 0.5 | 3.00 | 0.00 | 0.15 | 2917 | 2918.42 | -1.42 |
| 42 | 25.00 | 60.00 | 0.5 | 3.00 | 0.35 | 0.15 | 2932 | 2931.65 | 0.35 |
| 43 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.00 | 2022 | 2029.97 | -7.97 |
| 44 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.35 | 2716 | 2654.82 | 61.18 |
| 45 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3781 | 3724.28 | 56.72 |
| 46 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3673 | 3724.28 | -51.28 |
| 47 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3611 | 3724.28 | -113.28 |
| **48** | **25.00** | **60.00** | **0.5** | **3.00** | **0.15** | **0.15** | **3704** | **3724.28** | **-20.28** |
| 49 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3873 | 3724.28 | 148.72 |
| 50 | 25.00 | 60.00 | 0.5 | 3.00 | 0.15 | 0.15 | 3688 | 3724.28 | -36.28 |

[*] Runs in bold (2, 5, 10, 16, 23, 30, 33, 38, 40, 48) for testing; [1]Observed; [2]Predicted; [3]Residual

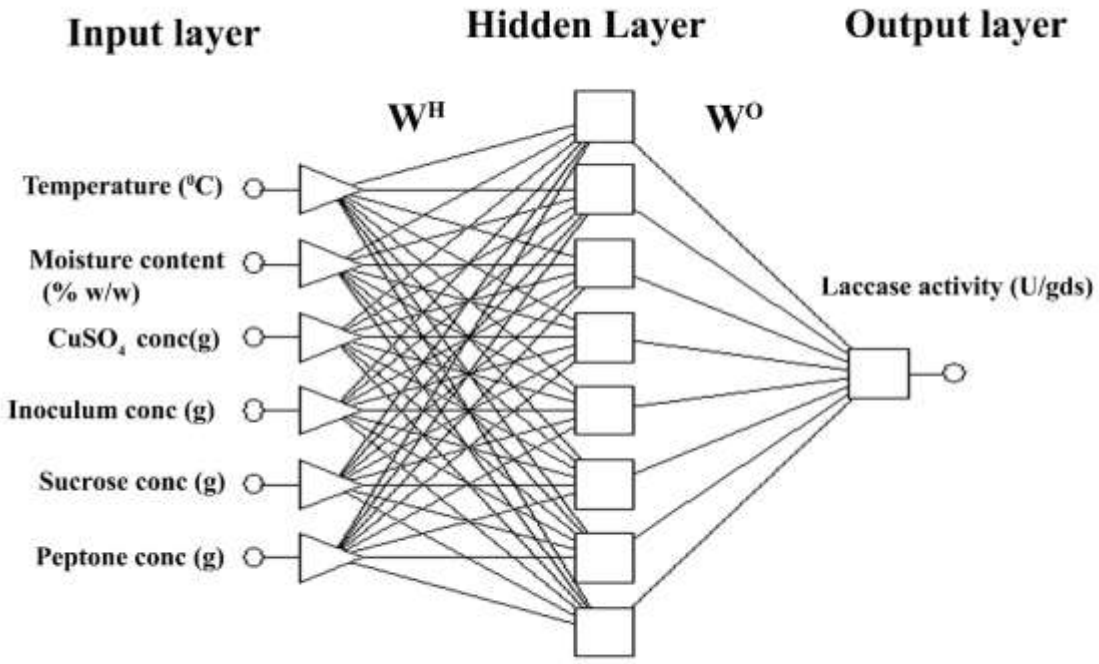


**Fig. 1.** Architecture of Neural Network constructed for optimization of laccase production in SSF

The effectiveness of the neural network prediction was evaluated by calculating the coefficient of $R^2$ value based on the measured and predicted outputs in the training and testing data. The calculated $R^2$ value was found to be 0.9963, specifying the model accuracy of the constructed ANN. The obtained $R^2$ value (0.9963) from ANN analysis was higher than the $R^2$ value (0.9617) obtained from the multiple linear regression of the same data. This indicates the superior quality of ANN for modeling the non-linear data when compared with traditional multiple regression analysis.

Figure 2 depicts the correlation between the experimental values and ANN predicted values. From this figure it can be observed that predicted values were concentrated near the diagonal line on the graph and no scattering points were noticed, which indicates the accuracy of the constructed ANN predictability.

Further, the certainty of the neural network was assessed based on MSE, RMSE, MAE, and MAPE of the training and testing data. The overall MSE (3016.4), RMSE (54.92), MAE (3.56), and MAPE ($8.9 \times 10^{-4}$) of the training data suggests that the constructed network is appropriate. This was further confirmed by testing data values of 260.94, 16.15, -7.12, and $-2.65 \times 10^{-3}$ for MSE, RMSE, MAE, and MAPE, respectively. Such a low magnitude of values confirms that the proposed neural network is a good approximation for modeling the laccase production data by isolated *P. ostreatus* PVCRSP-7. Similar magnitude values were reported by Sathish and Prakasham (2010) and Rao *et al.* (2008) for L-glutaminase and alkaline protease productions, respectively.

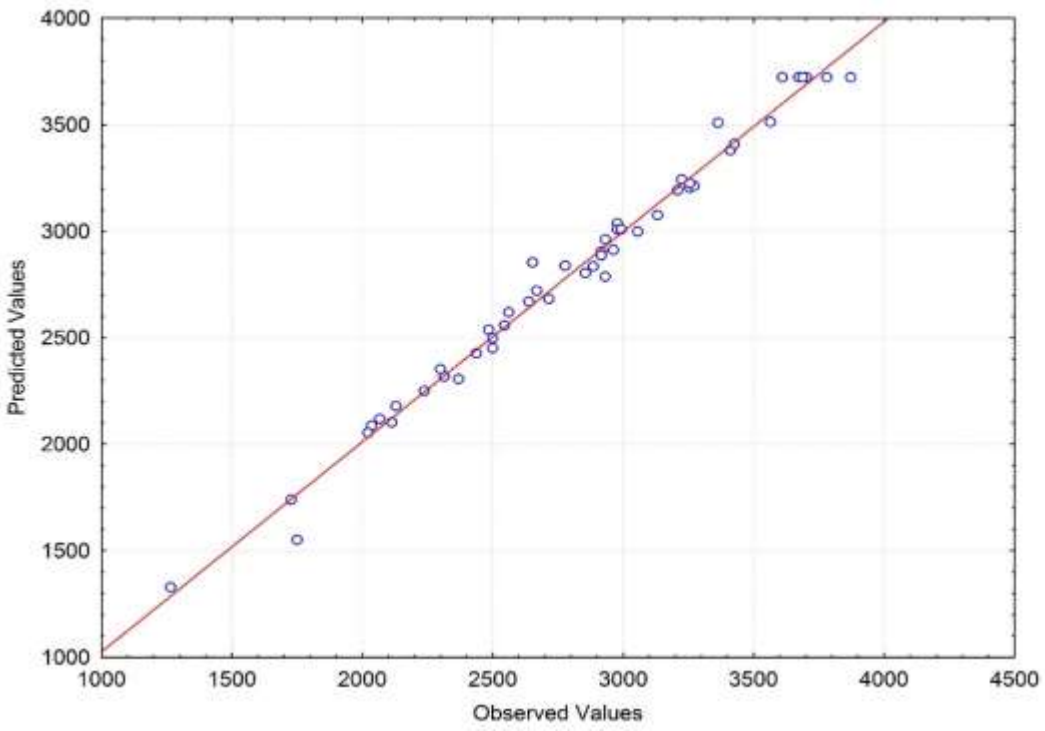


**Fig. 2.** Correlation graph of real and predicted laccase production data

## Interaction Influence of Selected Variables on Laccase Production

Figure 3 depicts the interactive influence of selected variables on lignolytic enzyme production by *P. ostreatus*. Figure 3a shows the interaction influence of moisture content and temperature. From this surface-contour plot it can be observed that moisture content above 50% and temperature below the 25 °C is favorable for higher amounts of laccase secretion by *P. ostreatus*. Figures 3b, 3d, and 3e indicate the interaction of $CuSO_4$ concentration with inoculum, glucose, and peptone; from all of these graphs it can be observed that $CuSO_4$ at 0.6 mgs is a suitable concentration for optimum secretion of lignolytic enzyme by isolated *P. ostreatus* PVCRSP-7. From Figs. 3b and 3c it can be seen that 2.5 to 3.5 gms of initial inoculum is optimum.

Figures 3c, 3d, and 3f depict that the concentration of additional glucose is regulated by other selected parameters. Observation reveals that 0.1 to 0.25 g of glucose is needed for enhanced lignolytic enzyme production by *P. ostreatus*. An additional nitrogen source (peptone) of 0.1 to 0.25 g is the most suitable concentration for the higher titer of laccase secretion by *P. ostreatus* (Figs. 3e and 3f).

## GA Optimization and Validation Studies

The optimum concentration of each chosen parameter was determined by using the GA, which was coupled to the ANN. The ANN generated output, weights and bias values, were used in the GA objective function. Among the 300 conditions generated by the GA, the 10 most suitable conditions were chosen, and experiments were performed at those conditions. The best conditions for higher laccase production were observed to be at a temperature of 24.3 °C, inoculum concentration of 2.7 g, moisture content of 65% (w/w), $CuSO_4$ of 0.55 mg/g substrate, glucose of 0.22 g/g substrate, and peptone of 0.18 g/g substrate. At these conditions, the laccase production was found to be 4244 U/gds, which is approximately a one-fold increment of enzyme production.

Kerem *et al.* (1992) and Membrillo *et al.* (2008) reported that the maximum laccase production in SSF by *P. ostreatus* was 0.03 U/g and 0.04 U/g using cotton stalks and sugarcane bagasse as substrates, respectively. Prasad (2005) reported that the highest enzyme activity after optimization by *P. ostreatus* 1804 was 2093.21 U/g, which has an extensive variation with others. The present study obtained a high laccase yield (4244 U/gds), which is closer to that of Prasad (2005).

The moisture content of the substrate plays a vital role in the growth of the microorganism as well as in controlling the excess temperature generated during the fermentation time (Laxmi *et al.* 2008). Figure 3a depicts the interaction of temperature with moisture content; more moisture and low temperatures in the studied range is favorable for greater lignolytic enzyme secretion by *Pleurotus ostreatus*. The obtained optimum temperature 24.3 °C and 65% moisture content values were closer to that of the Prasad (2005) results.

Copper is the key metal present in the laccase enzymes; the concentration of this metal in the media plays a critical role in fungal growth and secretion of enzymes (Tisma *et al.* 2012). In the present study, 0.55 mg of $Cu^{2+}$ was observed to be optimum for laccase production by *Pleurotus* sp. The obtained results are in agreement with the literature reports (Prasad 2005).

Based on preliminary studies, glucose and peptone were chosen as carbon and nitrogen supplements (data not shown). Figure 3f shows the interaction of glucose and peptone on the laccase production by *Pleurotus ostreatus*. From this figure it can be observed that both sources are needed in equal proportions; they do not conflict with each other. The obtained results are in accordance with Mikiashvili *et al.* (2006), who observed that the addition of peptone increases the maximum laccase yield from *P. ostreatus* 98 and *P. ostreatus* 108.

Statistical methods such as RSM and Taguchi facilitate the evaluation of the main and interaction effects of the factors. These methods have been employed to optimize the laccase production from white rot fungi (Levin *et al.* 2008; Teerapatsakul *et al.* 2007). Even though statistical methods reported better performance than the one at a time method, these methods also have some limitations. All statistical methods are limited by the number of factors, and these models determine the interaction influence based on the assumed polynomial models. To overcome these problems, artificial neural networks (ANN) and genetic algorithms (GA) have been utilized (Rao *et al.* 2008).

The GA approach is used in optimization and has the potential to optimize 12 to 14 variables at a time. Tisma *et al.* (2012) employed GA for optimization of the five components within 50 shake-flask experiments, where the highest laccase activity of 2,378 U/dm was achieved. In the present study, a hybrid of these artificial methods was employed. The program was set to ANN to model the experimental data, and the modeled data were subsequently subjected to optimization by GA.

There is no general rule for selecting the number of neurons in a hidden layer. It depends on the complexity of the system being modeled (Rao *et al.* 2008). According to Sathish and Prakasham (2010), a trial and error method is the best approach to determine the optimal number of neurons in the hidden layer. In the present study, eight neurons in the hidden layer gave the best predicted values. The obtained correlation coefficient ($R^2$ = 0.9963) indicates the proposed ANN model is adequate to model the experimental data. This was further confirmed by the MSE, RSME, and MAPE values of the training and testing data. The validation data also confirm that the GA predictions were trustworthy.

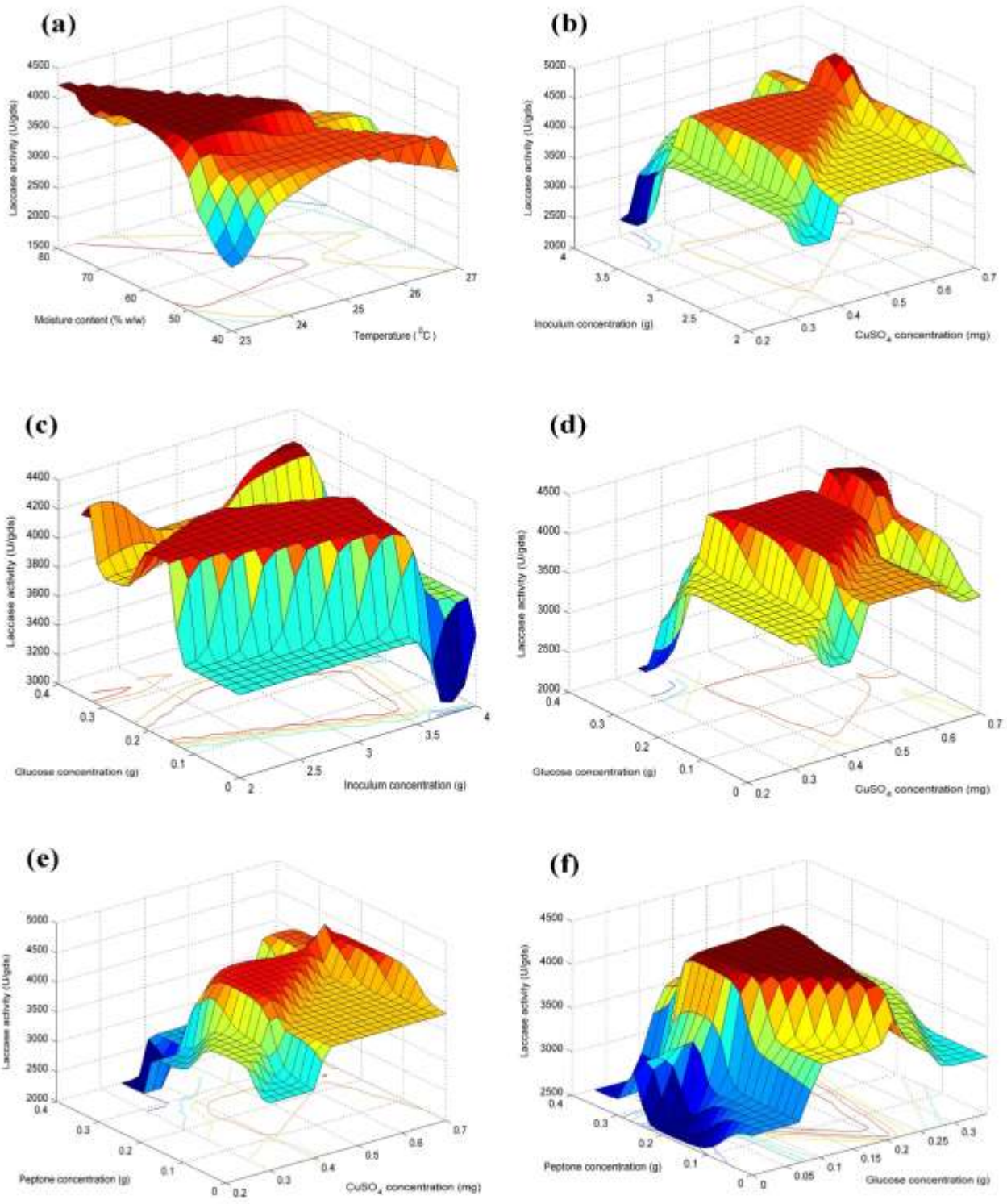


**Fig. 3.** Interaction influence of selected fermentation factors on laccase production (a) temperature *vs*. moisture content, (b) $CuSO_4$ concentration *vs*. inoculum concentration, (c) inoculum concentration *vs*. glucose concentration, (d) $CuSO_4$ concentration *vs*. glucose concentration, (e) $CuSO_4$ concentration *vs*. peptone concentration, and (f) glucose concentration *vs*. peptone concentration

## CONCLUSIONS

1. In comparison with other fungal strains, the isolated *P. ostreatus* PVCRSP-7 secreted higher amounts of laccase in SSF using black gram husk as a substrate.
2. The hybridization of the ANN-GA methods yielded the better optimum conditions. With the help of these methods, the laccase production was improved to 4244 U/gds which is nearly 100% improvement when compared with "one-at-a-time" method of optimization.

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