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Enzymatic degradation of pyridine raffinate using response surface and artificial neural network simulation

Manish Singh Rajput[1]*, Vinay Dwivedi[2]* & SK Awasthi[3]

[1]Department of Biotechnology, Dr. Ambedkar Institute of Technology for Handicapped, Kanpur, Uttar Pradesh, India
[2]Department of Biotechnology, Naraina Vidyapeeth Engineering & Management Institute Kanpur, Uttar Pradesh, India
[3]Harcourt Butler Technical University, Kanpur, Uttar Pradesh, India

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Pyridine is a heterocyclic aromatic compound present in pyridine raffinate, an organic discharge of the pyridine manufacturing industry. Besides pyridine, raffinate also contains formaldehyde, picolines and phenolics. Earlier, we isolated *Gamma proteobacterium* from timber soil for laccase production and optimized the involved process parameters. Here, we studied the optimization of process parameters for biodegradation of pyridine raffinate with the help of mathematical modeling [central composite design with response surface methodology (CCD-RSM) and artificial neural network (ANN)]. The results predicted ANN to be a better tool for optimization of pyridine raffinate degradation. CCD was used to develop the best fit second-order polynomial quadratic regression equation. Prediction of degradation percentage for pyridine raffinate was done using the equation which was found to be 71.60% at temperature 36.76°C, pH 7.45 and inoculum concentration 1.96 mL/10mL. The predicted response was experimentally validated in the wet lab to verify the degradation efficiency. The outcome was 65.76±2%, further confirmed by Gas Chromatography-Flame Ionization Detector (GC-FID). The result of GC-FID () data showed no trace of pyridine (Area 0%) which was reduced from initial area of 1.38% pyridine in raffinate sample.

Keywords: Backpropagation, Biodegradation, Central composite design (CCD), Industrial pollution, Laccase production, Organic pollutant, RSM

The increased industrialization without proper monitoring of effluents comprising toxic chemicals has led to serious environmental pollution. One such chemical is pyridine and its derivatives which are frequently found in the industrial and agricultural effluents. Due to its recalcitrant nature, the United States Environment Protection Agency (USEPA) has listed pyridine as a priority organic pollutant[1]. Utilization as a raw material in paint, dyes and pharmaceuticals along with its use in the alcohol denaturation process and to formulate products, such as medicine, vitamins, adhesives and in waterproofing of fabrics are some of the applications of pyridine[2,3]. Pyridine has carcinogenic properties, and hence an exposure limit of 5 ppm averaged over a 10 h work-shift has been recommended by Occupational Safety and Health Administration (OSHA), American Conference of Governmental Industrial Hygienists (ACGIH).

*Correspondence:
Phone: +91 98684 21746 (Mob.)
E-Mail: drvinay@yahoo.com (VD); msrbiotech@gmail.com (MSR)

Pyridine raffinate is a pale, toxic, obnoxious odour organic effluent released from different manufacturing industries, such as pharmaceuticals agrochemicals, food, latexes and others[4]. The raffinate consists of various heterocyclic aromatic hydrocarbon compounds, such as formaldehyde, phenolics and picolines along with pyridine and is highly alkaline (pH 12.0) and water soluble[5]. Among the various treatment methods investigated *viz*. sorption, zeolites and biodegradation[6], the biological treatment approach has been found to be most cost-effective. In this work, we opted for ex-situ bioremediation approach for degrading pyridine raffinate using bacterial extracellular enzyme laccase. The extensive applications of laccase have enhanced its industrial importance in areas like delignification of lignocellulosic material, waste detoxification and textile dye decolourization. The large amount of wastewater requiring treatment creates hindrance in the industrial application of enzyme assisted treatment technologies. High costs, limited operating stability, intolerance to unfavourable environmental conditions, and challenging recovery and recyclability are among the many other

drawbacks encountered while using soluble native enzymes for treatment[7]. Although laccase can be obtained from both bacteria and fungi, bacterial laccases are comparatively better for industrial use which could be attributed to their ability to perform in a broader range of temperature and pH and greater stability against different physical and chemical inhibitions.

In this study, we explored degradation of pyridine raffinate using laccase and to enhance the degradation process by optimizing the involved process parameters. Pyridine raffinate degradation was used as a model in this study for optimization of industrial degradation of pyridine and validation of the hypothesis was performed with central composite design (CCD). CCD is beneficial and efficient in providing information on the effects of variables involved in the experiment and overall experimental error in a minimum number of required runs (CCD-RSM). Box & Wilson developed response surface methodology (RSM) with the objective of improving yield from various industries[8]. The optimization of multiple parameters is performed in a stepwise manner by conducting a statistically designed experiment followed by determination of coefficient estimate, analysis of the response and finally checking the adequacy of the model.

In this study, we focused on optimizing the process parameter for degradation of pyridine raffinate using the diversity of isolated bacterial laccase to enhance the removal of pyridine on which only limited information is available. Furthermore, in order to screen the optimum process parameters, we used simulation tools like CCD-RSM[9-11] and ANN which is well known for optimization in biotechnology, life science, process industries[12]. We intend to select and screen the degradation parameters and further validate the parameters to apply in the industrial trial.

Materials and Methods

All chemicals used in this study were of analytical grade procured from Sigma Aldrich, Hi-media, BDH (British Drug House). The media and their ingredients were purchased from Hi-Media. Pure pyridine was used as a control sample and was procured from Thomas Baker.

Microorganism

Laccase producing bacteria was isolated from a soil sample in our previous study[13]. The bacteria were morphologically and biochemically characterized and identified as *Pseudomonas fluorescens* (Gamma proteobacterium).

Collection of pyridine raffinate sample

Plastic containers (Capacity 250 mL) were used to aseptically collect pyridine raffinate from M/S Jubilant Organosys Ltd, Gajraula (UP), India. The freshly collected pyridine raffinate samples were transparent, pale with pungent smell of formaldehyde, phenol, picoline, and pyridine.

Growth media

Solvents *viz*. Tris-Base, Phosphate buffer, EDTA and Propyl alcohol were obtained from Thomas Baker Chemicals Private Limited, New Delhi, India. Salts viz. Na_2HPO_4, $CaSO_4$, NaCl, $ZnSO_4$, $C_6H_5FeO_7$, $MnSO_4.H_2O$, $K_2Cr_2O_7$, $NaHCO_3$, NaH_2PO_4 and CH3COONa were obtained from Central Drug House, New Delhi, India and media components *viz*. agar, yeast extract, sucrose, glucose, tryptone, peptone were obtained from Sisco Research Laboratories Pvt. Ltd. (SRL), New Delhi, India. Guaiacol was procured from Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India. All reagents were of analytical grade and extra pure quality.

Laccase production media

The production of laccase was done in 250 mL Erlenmeyer flask, containing 50 mL of modified production media (g/l): 0.4 $CaSO_4.2H_2O$, 2.0 $MgCl_2.6H_2O$, 1.0 glucose, 5.0 Ferric citrate solution 0.01M, 0.5 Na_2HPO_4, 0.5 NaH_2PO_4, 0.5 NH_4NO_3, 0.5 K_2HPO_4, 3.0 yeast extract, 3.0 tryptone. pH was adjusted to 8.0 using 5N NaOH prior to sterilization (121°C, 15 lbs, 15 min). About 1% v/v inoculum (1×10^4 cells/mL)[14] was used for media inoculation and incubation was done at 30°C at 120 rpm for 5 days. After incubation was over, the culture broth was centrifuged at 10000 ×g for 10 min at 4°C and the supernatant was used as crude enzyme to measure laccase activity.

Process optimization

Experimental design and modeling

In this study, our aim was to obtain the optimum values of process parameters (temperature, pH and inoculum concentration) for efficient and optimized degradation of pyridine raffinate using CCD-RSM and ANN as statistical tools. RSM had been used as a prediction tool for process optimization[15]. As much as 20 experiments were conducted employing a three-level-three factor CCD taking into consideration the temperature (°C), pH and inoculum concentration (g/L) (Table. 1).

The influence of independent experimental factors and their interaction on pyridine raffinate degradation was predicted using a 2^3 rotatable CCD followed by RSM. Many researchers have successfully used CCD-

Table 1 — Range of parameters (independent variables) chosen for CCD

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Temp.	°C	Numeric	19.89	45.11	–1 ↔ 25.00	+1 ↔ 40.00	32.50	6.36
B	pH		Numeric	4.64	11.36	–1 ↔ 6.00	+1 ↔ 10.00	8.00	1.70
C	Inoculum conc.	g/l	Numeric	0.4887	3.01	–1 ↔ 1.00	+1 ↔ 2.50	1.75	0.6359

RSM for optimization and improved the degradation of PAHs[16].

The best fit second-order polynomial quadratic regression equation (Eq.1) was generated with the help of the response obtained through a CCD,

$$Y= \beta_0 + \beta_1A+ \beta_2B+ \beta_3C+ \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ \beta_{12}AB+ \beta_{13}AC+ \beta_{23}BC \quad \text{(Eq. 1)}$$

where, Y is the dependent variable (percentage of pyridine raffinate degradation); β_0 represents the offset value whereas β_1, β_2, β_3 are coefficients of linear terms; β_{11}, β_{22}, β_{33} are quadratic coefficients and β_{12}, β_{13}, β_{23} denote interaction coefficients. A, B, C represent the independent variables, *viz*., Temperature (°C), pH and inoculum concentration (g/L), respectively. Significance tests and analysis of variance (ANOVA) on each response were conducted to analyze the effect of the parameters and their interaction on the response in order to check the adequacy of the model. Design Expert 9.0 (State-Ease Inc., USA) was employed for the plotting three-dimensional surface plots. The value of α was calculated as 1.5 where α= 2k/4 (k=3, the number of variables). Table 2 represents the coded values of all independent variables and the experimental value of the only response variable Y (percentage of pyridine raffinate degradation) along with predicted values. The coefficients were calculated by Design-Expert 9.0.6.2.

Artificial neural network (ANN) *modeling*

A multilayered feed-forward ANN with error backpropagation (BP) was employed using MATLAB R2016a (MathWorks Inc., USA). Constant improvement of the network with proper mapping between input and output layers and error reduction is desired which is achieved by a strict learning scheme for proper training of the network[17,18]. The network was trained with Levenberg-Marquardt back-propagation algorithm (trainlm) in order to obtain the weights and biases. This algorithm typically requires more memory but less time. Minimization of error at each iteration during process optimization is most commonly done by a feed forward network with backpropagation[19]. The developed ANN architecture was used to optimize pyridine raffinate degradation using input neurons network topology. The number of hidden layer neurons was recognized by the training of several ANN topologies. The optimal one was selected on the basis of minimization of mean square error (MSE) and overall correlation coefficient (R) to improve the generalization ability of ANN topology. Overall, 20 experimental data points were used to construct and train the neural network model. About 70% of the overall data set was used for training the network model while 30% (15% + 15%) for testing and validation of the model. Training automatically stops as soon as improvement in generalization stops. This was indicated by an increase in the mean square error of the validation samples. The training of the neural network was performed until the MSE reaches a constant low value with accompanying overall correlation coefficient (R value) close to 1.

Table 2 — Experimental plan, range and levels of independent variables (A), (B) and (C)

Run	Temp. (°C)	pH	Inoculum (mL/ 10 mL)	Exp. removal (%)	RSM	ANN
1	25	6	1	25.63	20.99	25.74597
2	40	6	1	46.97	48.5	47.54515
3	25	10	1	42.97	43.72	40.81833
4	40	10	1	74.93	70.98	74.68941
5	25	6	2.5	30.92	35.13	31.58764
6	40	6	2.5	93.23	92.73	90.35077
7	25	10	2.5	36.36	35.08	38.33328
8	40	10	2.5	87.54	92.43	88.00668
9	20	8	1.75	16.36	17.06	14.15993
10	45	8	1.75	89.47	88.42	89.45637
11	32.5	4.5	1.75	47.05	46.81	47.22412
12	32.5	11.5	1.75	65.79	65.67	67.66411
13	32.5	8	0.5	37.46	41.34	37.69763
14	32.5	8	3.0	75.5	71.27	75.84445
15	32.5	8	1.75	61.55	57.55	58.06888
16	32.5	8	1.75	63.36	57.55	58.06888
17	32.5	8	1.75	48.11	57.55	58.06888
18	32.5	8	1.75	56.06	57.55	58.06888
19	32.5	8	1.75	58.73	57.55	58.06888
20	32.5	8	1.75	57.41	57.55	58.06888

Results and Discussion

Pyridine raffinate degradation:

Laccase was initially characterized by standard substrate guaiacol after which the best conditions were chosen to perform assay with pyridine raffinate. The bioremediation capability of the extracted enzyme was tested by analyzing the reduction in

optical density. Conical flasks (250 mL) each with 100 mL pyridine raffinate were inoculated with crude laccase enzyme and incubated according to CCD for evaluating degradation performance. Then, 5 mL of sample was taken aseptically at optimized time; 140 min and centrifuged (Elektrocraft Pvt. Ltd., MP400R, Mumbai, India) at 5000 rpm for 10 min[13]. The analysis of pyridine bioremediation was performed in terms of optical density reduction in the supernatant, observed at 501nm by a UV spectrophotometer (G Biosciences Pvt. Ltd., Noida, India) (Table 2).

The response expressed as percentage of pyridine raffinate degradation was calculated from Eq.2,

$$\% \text{ Degradation} = \frac{(C_0 - C_t)}{C_0} \times 100 \qquad \text{(Eq. 2)}$$

where C_0 is the initial optical density (OD) and C_t is the OD after time t (min).

Optimization of pyridine raffinate degradation

Central composite design (CCD) was employed for the experimental optimization of pyridine raffinate degradation. CCD model was used for experimental run, using temperature (A), pH (B), and inoculum concentration (C) as a variable parameter. The experimental run and the respective responses (i.e. degradation of pyridine raffinate in g/L) have been shown in Table 2. Error produced during the experiment was estimated before applying the RSM, ANN model for optimization.

Optimization by RSM modeling

The degradation of pyridine raffinate ranged between 16.36 and 93.23% removal. The experimental data was simulated using RSM for interaction analysis and response plot. The ANOVA model for pyridine raffinate degradation is shown in Table 3.

The model F-value of 35.86 implied model to be significant with only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicated the significant model terms which was A, B, C, AC, BC in this case. Model terms with values greater than 0.10 indicated insignificancy and if insignificant model terms are many (not counting those required to support hierarchy), model reduction may improve the model. The Lack of Fit F-value of 0.8295 implied an insignificant lack of fit relative to the pure error. There was 57.88% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good as it implies the model to fit. The predicted R^2 of 0.8621 was in reasonable agreement with the adjusted R^2 of 0.9429; the difference was less than 0.2. Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable and in this case a ratio of 20.961 indicated an adequate signal. Hence, the model was suitable to be used to navigate the design space.

Final equation regarding code factors

Pyridine raffinate degradation (%) = –97.46393 +2.48722Temperature +11.42961 pH+1.49961Inoculum–0.004250Temperature*pH + 1.33756Temperature*Inoculum –3.79583pH*Inoculum-0.030233 Temperature² –0.115346pH² –0.782526Inoculum² ……...(Eq. 3)

Predictions regarding the response for given levels of each factor can be made by using Eq. 3 in terms of actual factors where the levels should be specified in the original units for each factor. Fig. 1 depicts the interaction between the actual and predicted response. Further, the interaction graph and contour plot showing the relationship between two parameters keeping the remaining as constant is also presented.The interaction between inoculum concentration and pH is shown in

Table 3 — Analysis of variance (ANOVA) for pyridine raffinate degradation

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	8413.99	9	934.89	35.86	<0.0001*
A-temperature	6147.29	1	6147.29	235.80	<0.0001
B-pH	429.27	1	429.27	16.47	0.0023
C-inoculum	1081.39	1	1081.39	41.48	<0.0001
AB	0.0325	1	0.0325	0.0012	0.9725
AC	452.85	1	452.85	17.37	0.0019
BC	259.35	1	259.35	9.95	0.0103
A^2	41.68	1	41.68	1.60	0.2348
B^2	3.07	1	3.07	0.1177	0.7387
C^2	2.79	1	2.79	0.1071	0.7502
Residual	260.70	10	26.07		
Lack of fit	118.20	5	23.64	0.8295	0.5788**
Pure error	142.50	5	28.50		
Cor total	8674.69	19			

[*significant; **not significant]

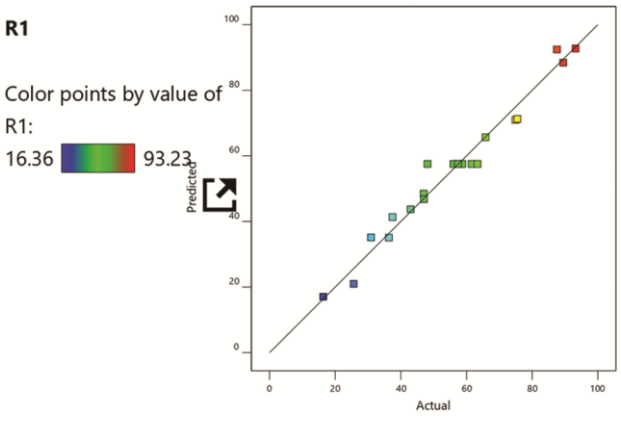

Fig. 1 —Relation between actual response and predicted response

Fig. 2A at a constant temperature 32.5 ℃. Similarly,the interaction between pH and temperature at a constant inoculum concentration of 1.75% is represented in Fig. 2B and interaction between inoculum concentration and temperature at constant pH 8 is represented in Fig. 2C. The optimum degradation of pyridine raffinate through RSM was evaluated at pH 7.45, temp 36.76°C and inoculum 1.96%. Fig. 3 depicts the optimum levels of the different parameters with the corresponding outcome for pyridine raffinate degradation.

Optimization by Artificial Neural Network (ANN)

Initially, for the ANN model, the input data was divided as training (70%), validation (15%) and testing (15%) and the performance of training is

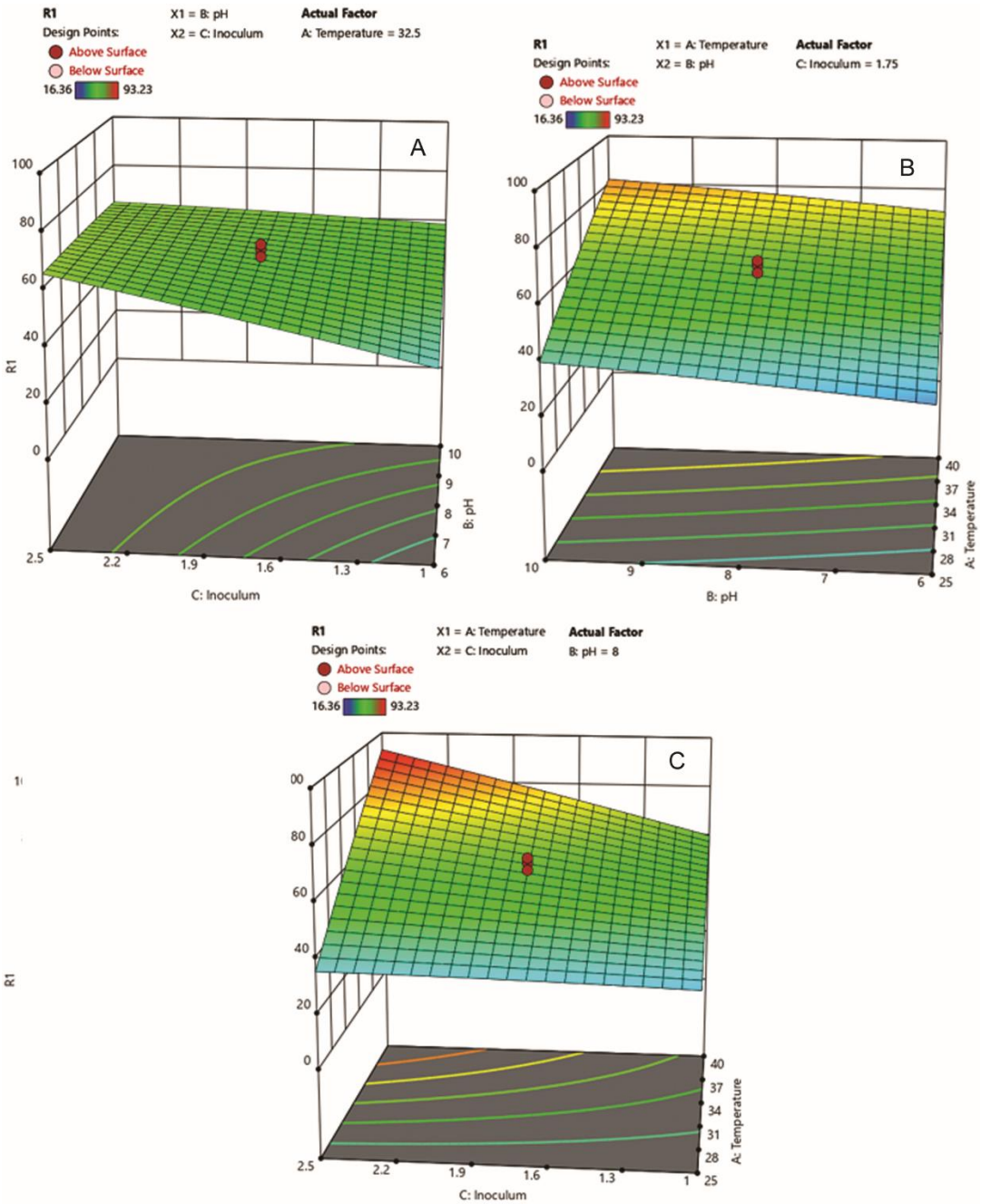

Fig. 2 — Contour graph interaction between (A) inoculum concentration and pH; (B) pH and temperature; and (C) inoculum concentration and temperature

depicted in Fig. 4A. The ANN model with suitable R^2 values of training (0.9848), validation (0.9981) and testing (0.9977) is represented in Fig. 4B. The overall model was best fit to a linear equation with R^2 value 0.99013 which was not close to R^2 value of RSM data set which was 0.9699. This indicates that accurate simulation for pyridine raffinate degradation (target) was provided by the developed ANN model and the experimental results were reproduced with greater precision. The quality of the data used to develop the ANN model was further estimated by error histogram plot which indicated most of the errors to range between -0.4276 to 0.3349 (Fig. 4C). The collection of more data points was necessitated in order to improve the model due to the presence of a large number of outliers in this model.

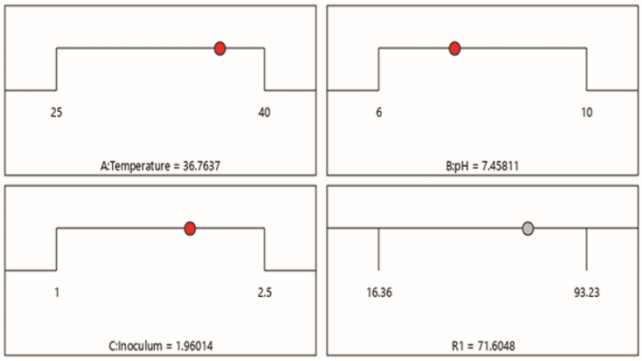

Fig. 3 — Optimum outcome of parameters

External validation by GC analysis

The GC-Flame ionization detector (GC-FID) was used to analyze the presence of pyridine along with other volatile and semi-volatile compounds in the raffinate and to validate the data regarding pyridine removal after microbial laccase treatment. Analysis of sample was performed by Agilent GC7890B with headspace 7697B (Agilent Technologies) to detect the compounds present using a GsBP-624 capillary

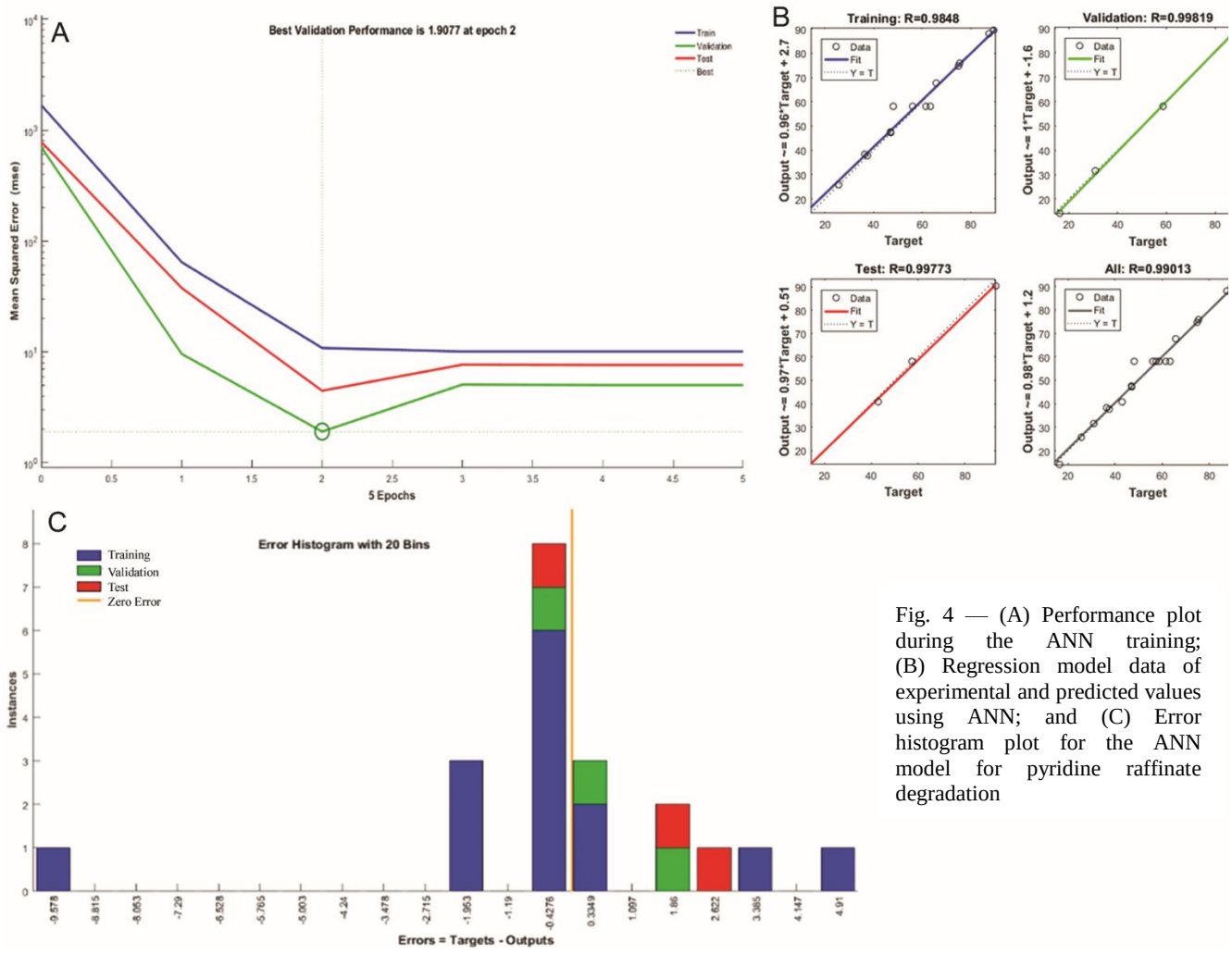

Fig. 4 — (A) Performance plot during the ANN training; (B) Regression model data of experimental and predicted values using ANN; and (C) Error histogram plot for the ANN model for pyridine raffinate degradation

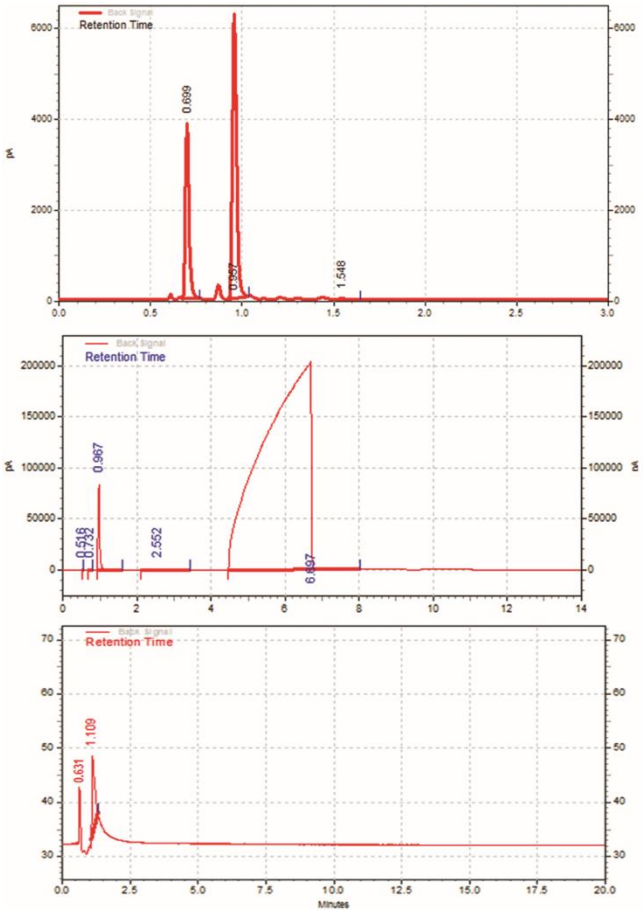

Fig. 5 — GC chromatogram of (A) pure pyridine analysis; (B) pyridine raffinate analysis; and (C) Analysis of pyridine raffinate after treatment by microbial laccase

column (30 m X 0.32 mm X 1.80 µm film thickness) (Agilent Technologies). Hydrogen was used as a carrier gas at a flow rate of 36 mL/min and the sample volume loaded was 1.0 µL. The column temperature was set at 100°C for 15 min. The volatile compounds present were detected by comparing their retention times depicted in the gas chromatogram to that of pure standard compound. The detection of the elements was done using a flame ionization detector. The chromatograms are shown in Fig. 5A-C. Fig. 5A indicates chromatogram for pure pyridine to be used as control with base material which depicts the peak with retention time and percentage (%) area for pyridine as 0.957 min and 65.10%, respectively; the retention time for base material was 0.699 min. (34.42%). The chromatogram in Fig. 5B depicts the different compounds present in the pyridine raffinate sample. The peak with retention time at 0.967 min. was confirmed as pyridine (1.38%) since the retention time

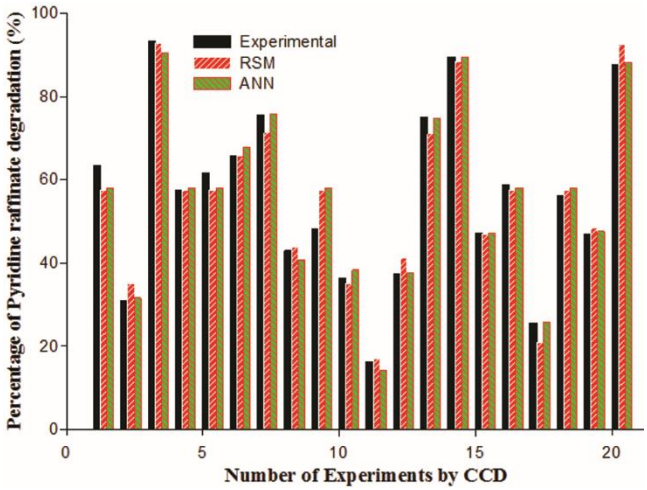

Fig 6 — Comparative analysis of experimental *vs*. CCD-RSM *vs*. ANN data

corresponded to that of pure pyridine peak in control. Another prominent peak was obtained at 6.697 min. (98.5%). The identification of the compounds associated with the other peaks is to be done in our future studies. The analysis of the pyridine raffinate sample after treatment with microbial laccase validated the removal of pyridine by the enzyme. The chromatogram in Fig. 5C showed two peaks at 0.631 min. (21.00%) and 1.10 min. (77.93%), respectively, none of which corresponded to the peak for pyridine in the chromatogram mentioned in Fig. 5B. This indicates the potential degradation of the pyridine in the raffinate sample after treatment by microbial laccase since 0% area was observed.

Comparing CCD-RSM and ANN data

The comparison of RSM and ANN data for validation has also been done in previous studies[15,20]. Fig. 6 depicts the comparison between CCD-RSM and ANN response where the data points of ANN response showed no overfitting. Also, the model was found to be statistically more suitable when compared to the RSM response. The experimental and predicted values of pyridine raffinate degradation by RSM and ANN are illustrated in Table 2. R^2 and mean square error (MSE) was employed as the basis for comparison of predictive capabilities of RSM and ANN models. R^2 values for the predicted model of RSM were found to be 0.9699 and that for the ANN model was 0.9901 whereas the MSE values for RSM and ANN models were 28.50 and 1.9077, respectively. One of the limitations with the RSM model is its ability to generalize data by only quadratic equations while ANN models demonstrate

higher predictive capability attributed to the non- linear polynomials of the system. The comparative predictive supremacy of ANN over experimental response has also been reported[21]. The selection of model type (RSM or ANN) mainly depends upon the type of data set to be used. RSM models are more appropriate in case of the limited number of components for obtaining the relation between input and output components and for sensitivity analysis[22]. However, on the other hand, ANN possesses the ability to learn and generalize the behaviour of any complex process and represents non-linearities in a much-rectified way[23].

Conclusion

In this study, we demonstrated degradation of the environmental pollutant pyridine raffinate by microbial laccase. We estimated the optimum process parameters required for adequate degradation of pyridine raffinate using central composite design with further analysis of the experimental outcome by coupling RSM and ANN model. ANN was found to be a better and improved tool for optimization of pyridine raffinate degradation. The predicted degradation of pyridine raffinate through the model was found to be 71.60% at temperature 36.76°C, pH 7.45 and inoculum concentration 1.96 mL/10 mL. The actual run had limitation of incubation temperature, therefore instead of 36.76°C, temperature was approximated at 37°C. At this condition, the actual degradation was determined by UV spectroscopy in terms of OD reduction and further validated by GC-FID. The optimum result was 65.76% which was further validated by GC-FID analysis to confirm the depicted area percentage (1.38%) of pyridine in the pyridine raffinate sample. After treatment by microbial laccase, the chromatogram showed no trace of pyridine (0% area) in the treated sample. Hence, microbial laccase could show potential in remediation of pyridine raffinate discharged into terrestrial and aquatic environment. Also, mathematically designed experiments (CCD-RSM/ANN) have great potential in the field of process optimization.

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Conflict of Interest

Authors declare no conflict of interests.

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