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Box-Benhken Approach for Media Optimization for Decolorization of Reactive Black 5h by Laccase from Isolated White Rot Fungus

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Abstract

A class of three level complete factorial design for the estimation of the parameters in a second-order model was developed by Box-Behnken method for decolorization of Reactive black 5H. Response surface methodology was found to be a useful tool in assessing the ability of the isolated white rot fungus on decolorizing azo dye molecule. The white rot fungus was isolated from wood decay sample, subjected for preliminary procedures of isolation and later identified by Phylogenetic analysis using basidiomycetes-specific internal transcribed sequences. Such a white rot fungus was identified to be *Trametes hirsuta* and was studied for its efficiency of laccase enzyme on decolorization of Reactive black 5H by response surface methodology. Three factors such as pH, temperature and agitation were optimized. The experimental values were very closer to the predicted value with the lack of fit value as 0.0987 while the R value was determined to be 0.987554.

Keywords: Decolorization, Laccase, Media optimization, Phylogenetic analysis, Reactive black 5H, *Trametes hirsuta*

Introduction

Synthetic dyes are used widely in many industries such as paper, colored

photography, and textile industry. There are 10,000 types of dyestuff throughout the world and approximately $7x10^5$ tones of these are produced every year. A remarkable amount of the dyestuffs is lost during dyeing processes. These losses are mainly disposed off into aquatic environment by textile, dyeing industries and some others means [1]. Due to the low biodegradability of dyes, conventional effluent treatment systems are inefficient in treating waste water. These include physical and chemical methods. These chemical or physical–chemical methods are less efficient, costly, of limited applicability and produce wastes, which are difficult to dispose of. As a viable alternative, biological processes have received increasing interest owing to their cost, effectiveness, ability to produce loss sludge and environmental benignity [2]. Alternative approaches utilizing microbial biocatalysts to remove dyes in textile effluent offers potential advantages over conventional processes due to minimal impact on the environment and cost effectiveness [3]. The capacity of fungi to reduce azo dyes is related to the formation of exoenzymes such as peroxidases and phenoloxidases. Peroxidases are hemoproteins that catalyse reactions in the presence of hydrogen peroxide. Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyse the oxidation of phenolic and other aromatic compounds without the use of cofactors [4]. The ligninolytic enzymes of white-rot fungi (e.g., Mn peroxidases, E.C. 1.11.1.13; lignin peroxidases, E.C. 1.11.1.7 and laccases, E.C.1.10.3.2) are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates [5] but also in the degradation of various xenobiotic compounds [6] including dyes [7]. Laccases (*para*-benzenediol:oxidoreductase) are glycosylated multi-copper oxidases widely distributed among higher plants, insects, bacteria and fungi.

Selection of experimental conditions providing increasing ligninolytic enzymes production, more than fungi growth, is very important for industrial applications and a few studies have been done to find a good experimental strategy [8].

The outstanding interest of the present study was to isolate the white rot fungus from decay wood sample which could degrade azo dye and to optimize the media conditions for its best decolourisation. The factors included for optimization of decolorization were pH, temperature and agitation. Response surface methodology was used for optimization which assessed the relationship between the variables and the activity of laccase.

Materials and methods

Sample collection, media and culture maintenance

Natural sample such as decay wood was collected from various parts of Hosur, Tamil Nadu, India, in sterile plastic covers and were brought to the laboratory without exposing to the external environment further. Potato dextrose agar was used in the beginning of the study to isolate fungi from decay wood sample. Malt extract (ME) agar was used for further study on the isolated fungi. The fungi culture was maintained on ME agar through periodic culturing at 30^0C. Mycelial plugs (5 mm in diameter) from seven days old culture were inoculated into ME liquid medium (three plugs per 100 ml in 500-ml Erlenmeyer flasks). After incubation for 7 days at 30°C

with agitation (80rpm) the culture was used for the study. ME medium was used as a blank in every assay.

Dyes used and isolation of white rot fungi

Reactive dyes are the dyes, which are mostly used in the textile industries. The following dyes were selected for the study- Reactive Black 5H and Direct Blue 71 (Sigma Chemicals, Mumbai, India). 0.2% concentration of both the dyes was used for screening of white rot fungi. Screening test of white rot fungi by solid state decolorization studies was carried out based on the method of Novotny et al [9]. The second phase of screening of white rot fungi was carried out based on similar method performed by Sathiya moorthy *et al*. [10]. This method of aqueous batch decolorization was performed with three different concentrations of both the dyes (25, 50 and 75 mg/l).

Decolourisation experiments

Decolorization assay was performed by incubating known concentration of dye with the media at appropriate pH and temperature. Dye decolourisation was expressed in terms of percentage calculated according to the following equation [11]:

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is an absorbance at Λ_{max} at time 0 and A_t is an absorbance at Λ_{max} of each dye after each time intervals.

Effect of pH and temperature on the production of laccase

To determine the optimum pH, temperature and agitation for laccase production by the isolated fungus, one variable at a time method was employed. The isolated fungi was grown in the malt extract medium adjusted with various pH from 3.0 to 11.0, (citrate buffer, acetate buffer, phosphate buffer, Tris–HCl buffer, carbonate–bicarbonate buffer) and at different temperature i.e., 10, 20, 30, 40, 50^0C. Laccase enzyme assay was done by the method explained by Srinivasan *et al.* [12].

Response surface methodology and central composite design

An RSM consists of an empirical modeling system that evaluates the relationship between a group of independent variables and observed responses [13]. This optimization process involves three major steps: (i) performing statistically designed experiments, (ii) estimating the coefficients in a mathematical model, and (iii) predicting the response and checking the adequacy of the model [14]. Response surface methodology (RSM) using central composite design (CCD) was applied to model the decolourization process since the conventional method of optimization, "one factor at a time" approach is laborious, time consuming and incomplete. This approach examines the efficient variation of important components with possible interactions; higher order effects and determines the optimum operational conditions

[15]. To understand the effect and interactions of variables on decolorization, a CCD with three variables at three levels were performed. The variables selected were pH, temperature and rate of agitation of the medium under study. Each variable was studied at three different levels (-1, 0, +1) for pH (5.5, 6.0, 6.5), temperature (25, 30 and 35°C) with agitation (60, 80 and 100 rpm). Experimental design included 17 flasks, with triplicates having all three variables at their central coded values. Experiments were performed in 100 ml conical flask with 50 ml malt extract broth containing 0.5% Reactive black 5H inoculated with the isolated fungi. Upon completion of experiments, percentage decolorization was calculated after 7 days of incubation as a dependent variable or response Y. Mathematical relationship of response Y and independent variable X (pH, temperature and agitation) was simulated by quadratic model equation as

$$Y_i = a_0 + \varepsilon a_i X_i + \varepsilon a_{ii} X_{ii}^2 + \varepsilon a_{ij} X_i X_j + e$$

Where, Y_i response or percentage decolorization at 596 nm for Reactive black 5H ; a_0, constant coefficient; a_i, i^{th} linear coefficient; a_{ii}, i^{th} quadratic coefficient; a_{ij}, different interaction coefficients; X_i X_j, coded independent variables related to factors; and e, error of model [16].

The CCD was applied using Design Expert Software, 8.0 trial version.

Results and Discussion

Screening and selection of white rot fungi

In the present study an attempt was made to isolate white rot fungi which could degrade azo dye efficiently from natural sample. About seven fungi could grow on potato dextrose agar medium. Fungi which could resist the presence of azodye in the medium could only grow on the medium and hence isolation was done on the medium with azodye. Malt extract agar was used for the selection of white rot fungi because the acidic nature of the medium would favor the growth of white rot fungi than any other fungi. Two fungi had exhibited good growth on MEA containing azo dye and the diameter of the zone of clearance in solid state fermentation was calculated after seven days of incubation at 30°C which was reported in Figure 1.

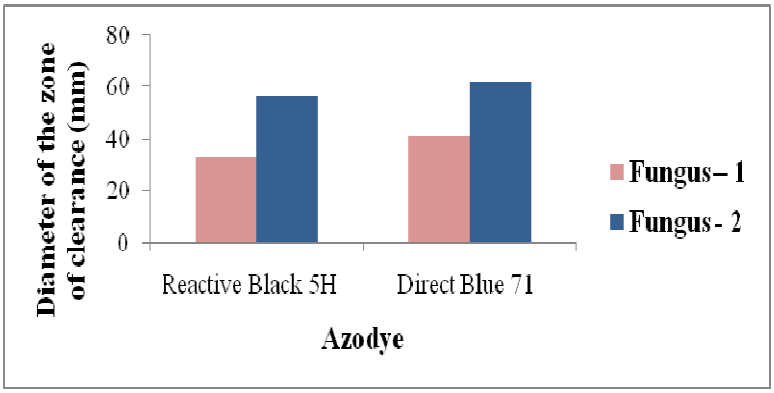

Figure 1: Solid state decolorization study of the two isolated white rot fungi.

Aqueous batch decolorisation study

When a particular concentration of sucrose or glucose provided, the decolorization or color removal process by white rot fungus was enhanced [12]. Hence the fungi isolated on MEA plate were assessed for their efficiency in decolourising synthetic azo dye molecules in a medium containing glucose.

There was significant disappearance of color from fourth day onwards and hence decolorization was assessed after 4 and 7 days of incubation period. Table 1 gives the percent decolorisation of Reactive Black 5H and Direct blue 71 by the two isolated fungi after 4 and 7 days of incubation. From the table it is evident that the dye Direct Blue 71 was better decolorized than Reactive Blue 5H by both the fungi. Fungus 2 was efficient in decolorizing Direct Blue 71 and exhibited best decolorization with 75 mg/L. Azodye molecules were best decolorized at their maximum concentration of 75 mg/L by both the fungi. A remarkable increase in the percent decolorisation of both the dyes was noticed after seven days of incubation period when compared to the per cent decolorisation after four days of incubation at all concentrations by both the isolated fungi. The decolorization efficiency was more with fungus 2 than with fungus 1 for both the dyes at all concentrations.

Table 1: Aqueous batch decolorization by the two isolated fungi.

Dye / Concentration (mg / L)	Per cent decolorization by isolated fungi			
	Fungus – 1		Fungus – 2	
	Post incubation periods (days)			
	4	7	4	7
Reactive Black 5H				
25	19	41	22	53
50	27	55	38	61
75	31	64	43	72
Direct Blue 71				
25	22	51	27	62
50	31	63	35	74
75	39	72	46	85

Similar work on five commercial and three wild types of *Pleurotus* species was performed by Biyik *et al.,* [17] with different concentrations namely 20, 50, 100, 200 and 500 mg/L of Cibacron Black W-NN. Swamy and Ramsay [18] evaluated the dye decolorizing ability of five species of white rot fungi with the similar methods of solid state decolorisation and aqueous batch decolorisation studies.

White rot fungi can completely mineralize lignin and a wide variety of aromatic compounds. These compounds include xenobiotic pollutants like polycyclic aromatic

hydrocarbons. The degradation of these aromatic compounds by white rot fungi depends on the production and secretion of lignin peroxidase, manganese-dependent peroxidase, and laccase. These are the key components of lignin degrading enzyme systems [19]. One of these enzymes is laccase and the substrates oxidized by laccase include ortho-, para-, diphenol, and aromatic compounds containing hydroxyl and amine groups [20].

The results of preliminary screening procedures showed better efficiency in azo dye degradation by fungus – 2. This was selected for further study and was identified by lacto phenol cotton blue test followed by basidiomycete – specific Internal Transcribed Sequence primers [21] (result not shown here). The most efficient fungus was identified to be as *Trametes hirsuta* by Phylogenetic analysis.

Ligninolytic fungi are able to degrade numerous aromatic organic pollutants via oxidative mechanisms [22]. A correlation between dye decolorization and the production of ligninolytic enzymes was implied by the work of Wesenberg and co-workers [23]. The ligninolytic enzymes are extracellularly excreted by the fungi to initiate the oxidation of substrates in the extracellular environment of the fungal cells [24]. Essential extracellular enzymes involved into the degradation of lignin in wood and recalcitrant pollutants (i.e. azo dyes) in the environment are laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP) [25]. Hence, one of the enzymes – laccase, responsible for azo dye decolorization was focused in this study.

Response surface methodology and central composite design

The results of one variable at a time study exhibited the pH and temperature for maximum production of laccase by the isolated white rot fungus to be 6 and 30°C respectively (results not shown in detail). Depending upon this preliminary study central composite design was made to optimize the conditions for maximum decolorization of azo dye by laccase. Hence the conditions selected for response surface methodology were pH (5.5, 6.0 and 6.5), temperature (25, 30 and 35°C) with agitation (60, 80 and 100 rpm). Since the three factors could influence the enzyme production there by its function, these were selected for the study with the response percent decolorization. The three factors under study for % decolorization showed appreciable amount of influence and the values of % decolorization ranged between 52 to 79 (Table 2). There was maximum decolorization when all the three parameters were 0 in the coded values (Run 6) and almost the same value was obtained in run 2 & 16. Least value 52 was obtained in run 9 when all the parameters were -1 in the coded value which implicated that any of the three conditions when dropped below the optimum could drastically reduce the % decolorization. But very good % decolorization (about 70%) was exhibited by the isolated *T.hirsuta* when at least any of the two conditions under study were optimum (0 in coded value) while good % decolorization (about 60%) was observed when at least any of two parameters were more than the optimum condition (1 in coded value).

Table 2: Full factorial central composite design for decolorization of Reactive Black 5H with actual and predicted values.

Run	pH	Temperature (°C)	Agitation (rpm)	% decolorization	
				Experimental value	Predicted value
1	1	0	0	71	71.79
2	0	0	0	78	78.21
3	-1	-1	1	62	60.98
4	0	1	0	73	71.79
5	0	0	1	71	72.99
6	0	0	0	79	78.21
7	-1	1	1	60	60.38
8	1	-1	1	60	59.38
9	-1	-1	-1	52	52.68
10	1	1	1	59	58.28
11	0	0	-1	70	68.19
12	-1	0	0	71	70.39
13	1	-1	-1	58	56.18
14	1	1	-1	56	56.98
15	-1	1	-1	52	52.58
16	0	0	0	78	78.21
17	0	-1	0	71	71.79

The model F-value of 61.71 (Table 3) implies the model is significant. There is only a 0.01% chance that a “Model F-value” this large could occur due to noise. Values of "Probability > F" less than 0.0500 indicate model terms are significant. In this case C, AC, A_++2_+-, B_++2_+-, C_++2_+- are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 9.42 (Table 4) implies there is a 9.87% chance that a "Lack of Fit F-value" this large could occur due to noise. The experimental values were very close to predicted values indicating the model was highly reliable. This is the reason for the lack of fit value to be very low.

Table 3: ANOVA for the quadratic equation of Design Expert 8.0 for the decolorization of Reactive Black 5H with three factors: pH, temperature and rate of agitation.

Source	Sum of squares	df	Mean square	F value	p-value (prob > F)
Model	1298.575	9	144.2861	61.71408	< 0.0001
A-pH	4.9	1	4.9	2.095828	0.1910
B-Temperature	0.9	1	0.9	0.384948	0.5546
C-Agitation	57.6	1	57.6	24.63667	0.0016

AB	0.125	1	0.125	0.053465	0.8238
AC	21.125	1	21.125	9.035586	0.0198
BC	0.125	1	0.125	0.053465	0.8238
A^2	135.812	1	135.812	58.08951	0.0001
B^2	100.3403	1	100.3403	42.91755	0.0003
C^2	155.5573	1	155.5573	66.53496	< 0.0001
Residual	16.36585	7	2.337978		
Lack of Fit	15.69918	5	3.139836	9.419507	0.0987
Pure Error	0.666667	2	0.333333		
Cor Total	1314.941	16			

Table 4: Lack of fit tests by Design Expert 8.0 for % decolorization as a function of pH, temperature and agitation.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Linear	1250.875	11	113.7159	341.1476	0.0029
2FI	1229.5	8	153.6874	461.0623	0.0022
Quadratic	15.69918	5	3.139836	9.419507	0.0987
Cubic	0.099178	1	0.099178	0.297535	0.6401
Pure Error	0.666667	2	0.333333		

The "Predicted R-Squared" of 0.8889 (Table 5 & 6) is in reasonable agreement with the "Adj R-Squared" of 0.9716. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 21.859 indicates an adequate signal.

Table 5: Model summary statistics of % decolorization of Reactive Black 5H as a function of three factors – pH, temperature and agitation.

Model Summary Statistics						
Source	Std.dev	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	9.81185	0.048215	-0.17143	-0.72004	2261.751	
2FI	11.09129	0.064471	-0.49685	-5.93141	9114.396	
Quadratic	1.529045	0.987554	0.971552	0.888889	146.1043	Suggested

Table 6: Analysis of variance (ANOVA) table for Response surface methodology.

Std. Dev.	1.529045	R-Squared	0.987554
Mean	65.94118	Adj R-Squared	0.971552
C.V. %	2.318801	Pred R-Squared	0.888889
PRESS	146.1043	Adeq Precision	21.85865

Final Equation in Terms of Coded Factors

$Y_1 = (78.21127) + (0.7A) - (0.3B) + (2.4C) - (0.125AB) - (1.625AC) - (0.125BC) - (7.11971A^2) - (6.11972B^2) - (7.61972C^2)$

The coefficient of determination of R^2 for the above equation was found to be 98.7 which indicated that the model is highly good.

The 3D surface plot and the Contour plot showing the effect of two variables with respect to the third variable as 0 are given in Figure 2, 3, 4, 5,6 and 7. These figures show that a very few experimental values are either above or below the theoretical value while the values are the same as expected values.

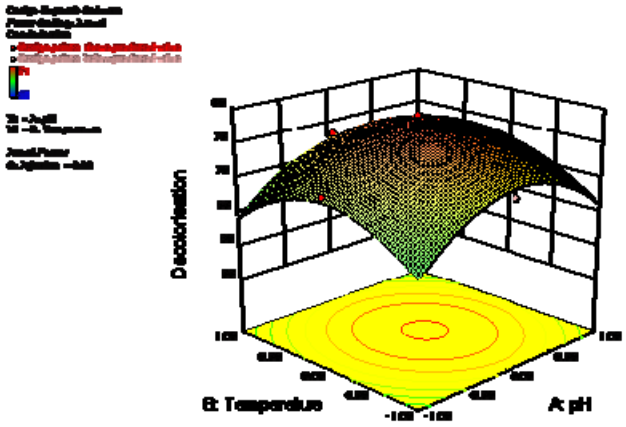

Figure 2: 3D surface plot showing effect of pH and temperature on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta.*

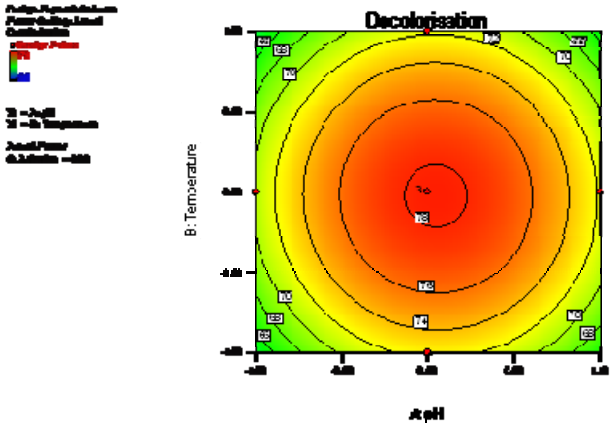

Figure 3: Contour plot showing effect of pH and temperature on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta.*

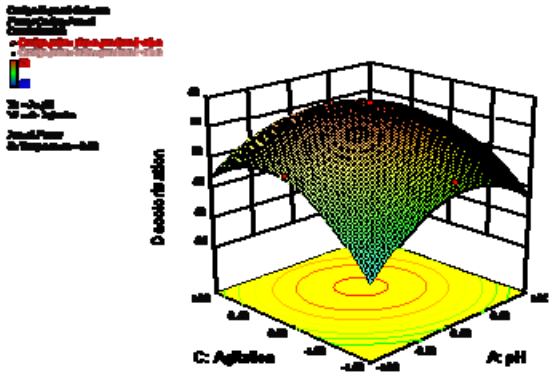

Figure 4: 3D surface plot showing effect of pH and agitation on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta.*

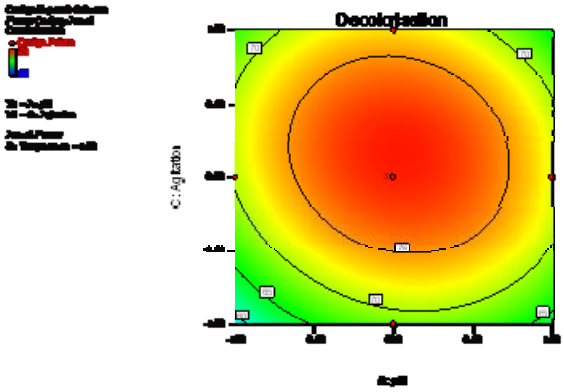

Figure 5: Contour plot showing effect of pH and agitation on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta*.

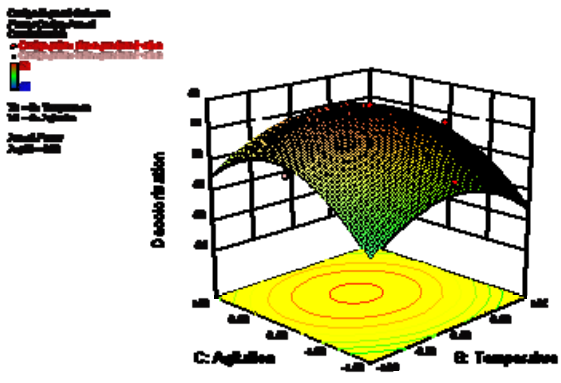

Figure 6: 3D surface plot effect temperature and agitation on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta.*

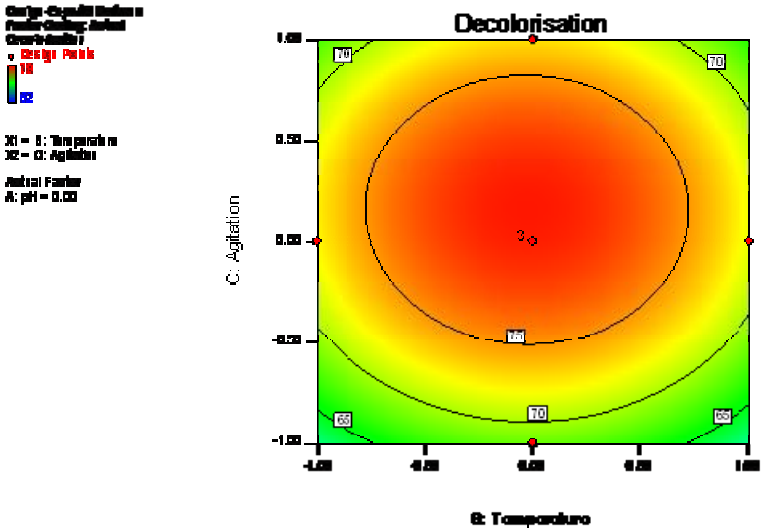

Figure 7: Contour plot showing effect temperature and agitation on % decolorization of Reactive Black 5H dye by isolated *T.hirsuta*.

Singh *et al.* [26] carried out RSM for alkalophilic laccase production from Gamma-proteobacterium JB for the variables namely pH, incubation time, agitation and $CuSO_4$ concentration. Experimental findings were in close agreement with the model predictions with the adjusted R^2 value of 0.98. Quaratino *et al.* [27] performed RSM for laccase production in *Panus tigrinus* with five variables namely glucose, nitrogen, Cu^{2+}, 2,5-xylidine and olive-mill wastewater.

Murugesan *et al.* [28] performed Box-Behnken design using RSM for the decolourization of the azo dye RB-5 using purified laccase from a white rot fungus *Pleurotus sajor-caju*. The four variables namely dye, enzyme, redox mediator concentrations and incubation time were optimised. The experimental values were in good agreement with predicted values and the model was highly significant, the correlation coefficient being 0.999.

Optimization of laccase production by *Coriolus versicolor* in solid state fermentation was performed by Mishra *et al.* [29]. The variables namely pH, temperature, moisture content, inducers, groundnut shell and cyanobacterial biomass were studied. Correlation coefficient (0.9758) indicated a good agreement between experimental and predicted values.

A central composite design was applied by Trupkin *et al.* [30] to optimize copper, veratryl alcohol and L-asparagine concentrations for *Trametes trogii* (BAFC 212) ligninolytic enzyme production in submerged fermentation. R^2 coefficient of determination was found to be 0.799. Revankar *et al.* (2007) [31] optimised the concentration of wheat bran, CuSO4, starch, yeast extract and moisture content for laccase activity by solid-state fermentation using an indigenously isolated white rot basidiomycete *Ganoderma* sp.

Conclusion

Since the industrial release of synthetic dyes in to the environment poses a major problem to all parts of the world, the present study gave an attempt to degrade the synthetic dye effectively by the isolated white rot fungus. The isolated fungus was found to be the best amongst the other fungi isolated by solid state and aqueous batch decolorization procedures. The isolated fungus was identified to be *Trametes hirsuta* by basidiomycete-specific ITS primers method. The study was emphasized to optimize the culture conditions such as pH, temperature and agitation for maximal decolorization of the azo dye molecule by response surface methodology. The optimum conditions were identified to be 80 rpm at 27°C at the pH value of 6.0 and the model was found to be highly significant with these conditions.

References

[1] Goksel Demir, H., Kurtulus Ozcan, Nese Tufekci and Mehmet Borat., 2007, Decolorization of Remazol Yellow RR Gran by white rot fungus *P.chrysosporium,* Journal of Environmental Biology, 28(4), pp. 813-817.

[2] Chen, K.C., Wu, J.Y., Liou, D.J., and Hwang, S.C.J., 2003, Decolorization of the textile dyes by newly isolated bacterial strains. J. Biotechnol., 101, pp.57–68.

[3] Hiroyuki, W., Kabuto, M., Mikuni, J., Oyadomari, M., and Tanaka, H., 2002, Degradation of water insoluble dyes by micro-peroxidase 11: an effective and stable peroxidative catalyst in hydrophilic organic media, Biotechnol. Prog., 18, pp. 36 – 42.

[4] Duran, N, Rosa, M.A., and Gianfreda L., 2002, Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review. Enzyme and Microbial Technology, 31, pp.907-931.

[5] Hatakka, A., 1994, Lignin-modifying enzymes from selected white rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev, 13, pp.125–135.

[6] Scheibner, K., Hofrichter, M., and Fritsche, W., 1997, Mineralization of 2-amino-4,6-dinitrotoluene by manganese peroxidase of the whiterot fungus *Nematoloma frowardii*. *Biotechnol. Lett.,* 19, pp.835–839.

[7] Moreira, M.T., Mielgo, I., Feijoo, G., and Lema, J.M., 2000, Evaluation of different fungal strains in the decolourisation of synthetic dyes. Biotechnol. Lett,. 22, pp. 1499–1503.

[8] Nowak, G., Matuszewska, A., and Nowak, M., 1998, Protein secretion and oxidase activities in idiophasic cultures of *Trametes versicolor.* In: 7th International Conference of Biotechnology in the Pulp and Paper Industry, pp.B131-134.

[9] Novotony, C., B. Rawal, M. Bhatt, M. Patel, V. Sasek and H.P. Molitoris, 2001. Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes. J. Biotechnol. 89: 113-122.

[10] Sathiya moorthi, P., S. Periyar selvam, A. Sasikalaveni, K. Murugesan and P.T. Kalaichelvan, 2006. Decolorization of textile dyes and their effluents using white rot fungi. African Journal of Biotechnology. 6 (4): 424-429.

[11] Sarnthima, R. and S. Khammuang, 2008, Laccase isozymes of *Pleurotus sajor-caju* culture on husk and bran of black sticky rice and their potential on indigo carmine decolourisation. African Journal of Biotechnology, 7 (20), pp.3731-3736.

[12] Srinivasan, C., D'souza, M., Boominathan, K., and Reddy, C. A., 1995, Demonstration of Laccase in the White Rot Basidiomycete *Phanerochaete chrysosporium* BKM-F1767, Appl. and Env. Microbio., pp. 4274–4277.

[13] Vasconcelos, A. D., Barbosa, A. M., Dekker, R. F. H., Scarminio, I. S., and Rezende, M. I. 2000, Process Biochemistry, 35, pp.1131–1138.

[14] Box, G.E.P., and Hunter, J.S., 1957, Multifactor experimental design for exploring the response surfaces. Ann. Math. Stat.,28, pp.195-8.

[15] Roriz ,M.S., Osma, J.F., Teixeira,J.A. and Couto, S.R., 2009, Application of response surface methodological approach to optimise Reactive Black 5 decolouration by crude laccase from *Trametes pubescens,* Journal of Hazardous Materials 169, pp.691–696.

[16] Dahiya, D., Singh,N., and Rana, J.S., 2009, Optimization of growth parameters of phytase producing fungus using RSM, Journal of Scientific & Industrial Research, 68, pp.955-959.

[17] Biyik, H., F. Kalyonc, E. Oryasin, N. Azbar, E. Kalmı and G. Basbulbul, 2009, Evaluation of wild and commercial types of *Pleurotus* strains for their ability to decolorize cibacron black WNN textile dye. African Journal of Microbiology Research 3(6), pp.325-329.

[18] Swamy J and Ramsay, 1999, The evaluation of white rot fungi in the decoloration of textile dyes. Enz. And Microbial tech. 24, pp.130-137.

[19] Xiao, Y., X. Tu, J. Wang, M. Zhang, Q. Cheng, W. Zeng, and Y.Shi. 2003, Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete Trametes sp. strain AH28-2. Appl. Microbiol. Biotechnol. 60, pp.700-707.

[20] Clutterbuck, A.J., 1990, Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen. Microbiol. 136, pp. 1731-1738.

[21] Prewitt, L.M., S.V. Diehl, T.C. McElroy and W.J. Diehl, 2008. Comparison of general fungal and basidiomycete-specific ITS primers for identification of wood decay fungi. Forests Products Journal. 58(4), pp. 66-71.

[22] Adosinda, M, Martins, M., Ferreira, I. C., Santos, I. M., Wueiroz, M. J., and Lima, N., 2001, Biodegradation of bioaccessible textile azo dyes by *Phanerochaete chrysosporium.* Journal of Biotechnology, 89, pp.91-98.

[23] Wesenberg, D., Kyriakides, I., and Agathos, S.N., 2003, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnology Advances, 22, pp.161-187.

[24] Mester, T., and Tien, M., 2000, Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. International Biodeterioration & Biodegradation, 46, pp.51-59.

[25] Hou, H., Zhou, J., Wang, J., Du, C., and Yan, B., 2003, Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye, Process Biochemistry, 39, pp.1415-1419.

[26] Singh, G.,Ahuja, N,Sharma, P and Capalash, N., 2003, Response surface methodology for optimized production of an alkalophilic laccase from *Gammma-proteobacterium* JB. Bioresources, 4(2), pp. 544-53.

[27] Quaratino, D.,Ciaffi, M., Federici, E. and D'annibale, A., 2008, Response surface methodology study of laccase production in *Panus tigrinus* liquid cultures Biochemical Engineering Journal 39, pp. 236–245.

[28] Murugesan, K., Dhamija, A., Nam, I., Kim, Y. and Chang Y., 2006, Decolourization of reactive black 5 by laccase: Optimization by response surface methodology. Dyes and Pigments 75, pp.176-184.

[29] Mishra, A., Sunil Kumar and Sudhir Kumar, 2008, Application of Box-Benhken experimental design for optimization of laccase production by *Coriolus versicolor* MTCC 138 in solid-state fermentation, J. of Scientific & Industrial Research, 67, pp.1098-1107.

[30] Trupkin, S., Levin, L., Forchiassin, F. and Viale, A., 2003, Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology, J Ind Microbiol Biotechnol 30, pp. 682–690.

[31] Revankar, M. S., Desai, K.M. and Lele, S. S., 2007, Solid-state Fermentation for Enhanced Production of Laccase using Indigenously Isolated Ganoderma sp. Appl Biochem Biotechnol (2007) 143, pp.16–26.