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Pleurotus ostreatus Laccase Decolorization of Remazol Brilliant Violet 5R Dye: Statistical Optimization and Toxicity Studies on Microbes and its Kinetics

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Abstract

Introduction: Nowadays, enzyme-based removal of hazardous dyes that pollute the environment has been considered as a substitute and eco-friendly method compared to the physical and chemical method. The present study was conceived in order to obtain the optimal condition for laccase-mediated (purified from the *Pleurotus ostreatus* PKN04) decolorization of Remazol Brilliant Violet 5R dye; a mono-azo dye, using the central composite design of response surface methodology (RSM).

Materials and Methods: The design of experiment was suggested with 6 variables including pH, temperature, incubation time, agitation, dye concentration, and enzyme concentration, which were applied in order to optimize the decolorization process. The kinetic and energetic factors of laccases for the enzymatic removal of Remazol Brilliant Violet 5R dye was investigated.

Results: Decolorization of Remazol Brilliant Violet 5R was maximally 95.72%, which had occurred at 6.0 pH, 40°C temperature, 60 minutes incubation time, 50 rpm agitation, 50 ppm dye concentration, and 100 IU/mL enzyme concentration. The obtained results of kinetic introduced the laccase-catalyzed decolorization of Remazol Brilliant Violet 5R as an endothermic reaction with K_m and V_{max} values of 0.801 mM and 387 mM/mg/min, respectively. In addition to the above results, the toxicity study against bacteria revealed that the toxicity of laccase-treated dye drastically reduced to the untreated dye.

Conclusions: The results of the present analysis reveal that the *Pleurotus ostreatus* laccase is an efficient biocatalyst for decolorization of synthetic dye Remazol Brilliant Violet 5R dye.

Keywords: Enzyme Catalysis, Optimization, Response Surface Methodology, Central Composite Design, Decolorization, Laccase

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Introduction

White-rot basidiomycetes are the most effective microorganisms to decolorize and degrade a wide range of dyes and pollutants. These fungi specifically attack ligninolytic compounds by producing oxidative enzymes, namely the peroxidases and laccases.[1] Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are a small collection of enzymes called the blue copper proteins or the blue copper oxidases along with the plant ascorbate oxidase and the mammalian plasma protein ceruloplasmin[3,4] among others. Laccase is an enzyme secreted by many of the lignin degrading basidiomycetes and has been reported as an essential enzyme for lignin degradation in fungi without per-oxidases.[5] This enzyme catalyzes the oxidation of a wide number of phenolic compounds and aromatic amines but its substrate range have been extended to non-phenolic compounds in the presence of low molecular mass compounds acting as mediators.[6,7] Most of the studies have been carried out with laccases from eukaryotes, principally with enzymes secreted by basidiomycetes being their distribution in prokaryotes more recently. Different industrial purposes for fungal laccases have been insinuated and they include paper processing, inhibition of wine decolouration, detoxification of ecological pollutants, oxidation of dye and their precursors, enzymatic translation of chemical intermediates, and production of chemicals from lignin. Before laccases can be commercially employed for potential applications, however, an inexpensive enzyme source needs to be made available.[8-10] Two of the most intensively studied areas in the potential industrial application of laccase are the delignification and pulp bleaching and the bioremediation of contaminating environmental pollutants.[11]

The response surface methodology (RSM) which is broadly utilized in optimization processes, implicates a compilation of useful mathematical and statistical skills for analyzing the

relationship between independent variables, responses, and their interactions through the construction of polynomial mathematical models which leads to time and cost saving.[12-14] In the present study, a central composite design of RSM as a very suitable design method was employed to acquire maximal removal of Remazol Brilliant Violet 5R dye supported by laccase. The kinetic parameters of laccase-facilitated dye removal process were investigated. The toxicity against different bacterial experiments were also instigated in order to assess the toxicity of laccase degraded dye.

Materials and Methods

Chemicals

Remazol Brilliant Violet-5R (RBV-5R) $C_{20}H_{16}N_3Na_3O_{15}S_4$ Molecular weight 735.58 and λ_{max} of 570 nm, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was purchased from Sigma-Aldrich (Bangalore, India). All chemicals and reagents had analytical grade purity. The extracellular laccase of *Pleurotus ostreatus* PKN04 was purified using various methods.[15]

Organism

Pleurotus ostreatus PKN 04 was isolated from the decomposed wood and leaf litters of Chennai forest and grown in Sabouraud dextrose agar (SDA). The organism was screened for the production of laccase by ABTS method, subcultured to obtain pure culture and identified using 18s rRNA sequencing method.[16]

Pleurotus ostreatus PKN 04 was grown in Malt Extract Medium (MEA) and the spores were washed and inoculated in the fermentation medium.[17]

Analysis of Enzyme Activity

The activity of the free laccase was assayed using 1 mM ABTS in distilled water as substrate. The reaction mixture consisting of 3 mL of 0.1 M acetate buffer, 4.5 pH containing enzyme was initiated by adding 0.3 mL of ABTS. Free enzyme by the addition of 10 seconds intervals at 420 nm Cary Varian UV/VIS absorbance measurements were taken by spectrophotometer. Ten beads, 2.7 mL acetate buffer and 0.3 mL of ABTS were incubated with shaking at 100 rpm for 5 minutes for immobilization laccase activity. To separate the beads at the end, the upper phase at 570 nm absorbance was measured. One activity unit of laccase was defined as the amount of enzyme required to catalyze 1 mmol/mL of substrate per minute.[18,19]

Dye Decolorization Experiments

After preparation of dye solution (concentration range of 50–150 mg/L) in citrate-phosphate buffer (0.1 M, pH range of 3.0–8.0), the partially purified laccases (final activity of 1–2.5 U/L) were added to the reaction mixture and incubated at desired temperature (30–50°C) for 30–90 minutes. It was followed by measuring the absorbance of the taken samples using a UV/visible spectrophotometer at maximum absorbance of applied dye (570 nm).[20]

Decolorization percentage was then calculated using the following equation:

$$Decolorization = \frac{(A_i - A_t)}{A_i} \times 100$$

A_i is the initial absorbance of the reaction mixture and at is the absorbance after incubation time. The reaction mixture with the heat-inactivated enzyme was formulated (negative control) and incubated at similar conditions. All trails were performed in duplicates and the means of decolorization percentages were reported.

Response Surface Methodology

The central composite design has been developed to select the design points as the design of experiments (Table 1) that minimizes the related variances and analyzed using FT-IR.[21]

Dye Removal Kinetics

Kinetics of Decolorization

After performing decolorization in the presence of different concentrations of dye (10–500 µM) at an ideal temperature and pH, the velocity for different concentrations of dye were determined. The MM curve was then drawn by plotting the attained initial velocity verses dye concentrations. Calculation of K_m and V_{max} values were performed by fitting the data to the Lineweaver-Burk plot, resulting of the Michaelis-Menten plot conversion using Hyper 32 software.[22-25]

Toxicity Studies on Microbes

A series of testing were performed to evaluate toxicity of the synthesized metabolite following laccase therapy. The pre-incubated bacterial culture *Staphylococcus aureus* MTCC 740, *Bacillus subtilis* MTCC 441 and *E. coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 741, and *Salmonella typhi* MTCC 3124 was prepared by incubating each bacterial strain in Muller-Hinton broth at 37°C and 150 rpm to reach the OD_{620} of 0.2. Consequently, the untreated dye solution (final concentration of 100 mg/L) and the sample obtained from enzymatic treatment of applied dye (performed at the

Table 1. Design of Experiment

Factor	Low		High		Mean	SD
	Coded	Actual	Coded	Actual		
A - pH	-1	4	1	8	6	1.790141
B - Temperature	-1	30	1	50	40	8.950703
C - Incubation Time	-1	30	1	90	60	26.85211
D - Agitation	-1	0	1	100	50	44.75352
E - Dye concentration	-1	50	1	150	100	44.75352
F - Enzyme concentration	-1	1	1	2.5	1.75	0.671303

optimized condition) was separately added to the prepared bacterial culture media and incubated at 37°C. Changes in the OD_{620} of each bacterial strain were then recorded every 2 hours for 10 hours. A negative control (cultivated bacterial strain in the absence of dye) was designed for each experiment.[26,27]

$$The\ precentage\ of\ grwoth\ inhibition = \frac{(1 - OD_{620}Sample)}{OD_{620}Control}$$

Statistical Analysis

The data were obtained from duplicate experiments unless mentioned particularly and the experimental results were expressed as mean ± standard deviation.

Results and Discussion

Isolation and Identification of Organism

The isolated organism was identified using the macroscopic and microscopic method. The microscopic features are as follows: spores 8-10.5 x 3-3.5 µ; smooth; cylindrical to narrowly kidney-shaped may be an epitype collection of *Pleurotus ostreatus* PKN04.[28]

Optimization of Factors using Response Surface Methodology

Optimization was done using the Central Composite Design (CCD). Results show significance as the R^2 value and the R^2 adjusted value are close enough and the error ration is less than 0.05. The interaction of the variables was shown in Figure 1. The statistical outcomes (actual and predicted results) for the ideal equation was displayed in Table 2. The F value quantifies the difference of the data about the mean. Also, the *P* value functions as a tool for examining the importance of every coefficient. The predicted R^2 value is logically accord to

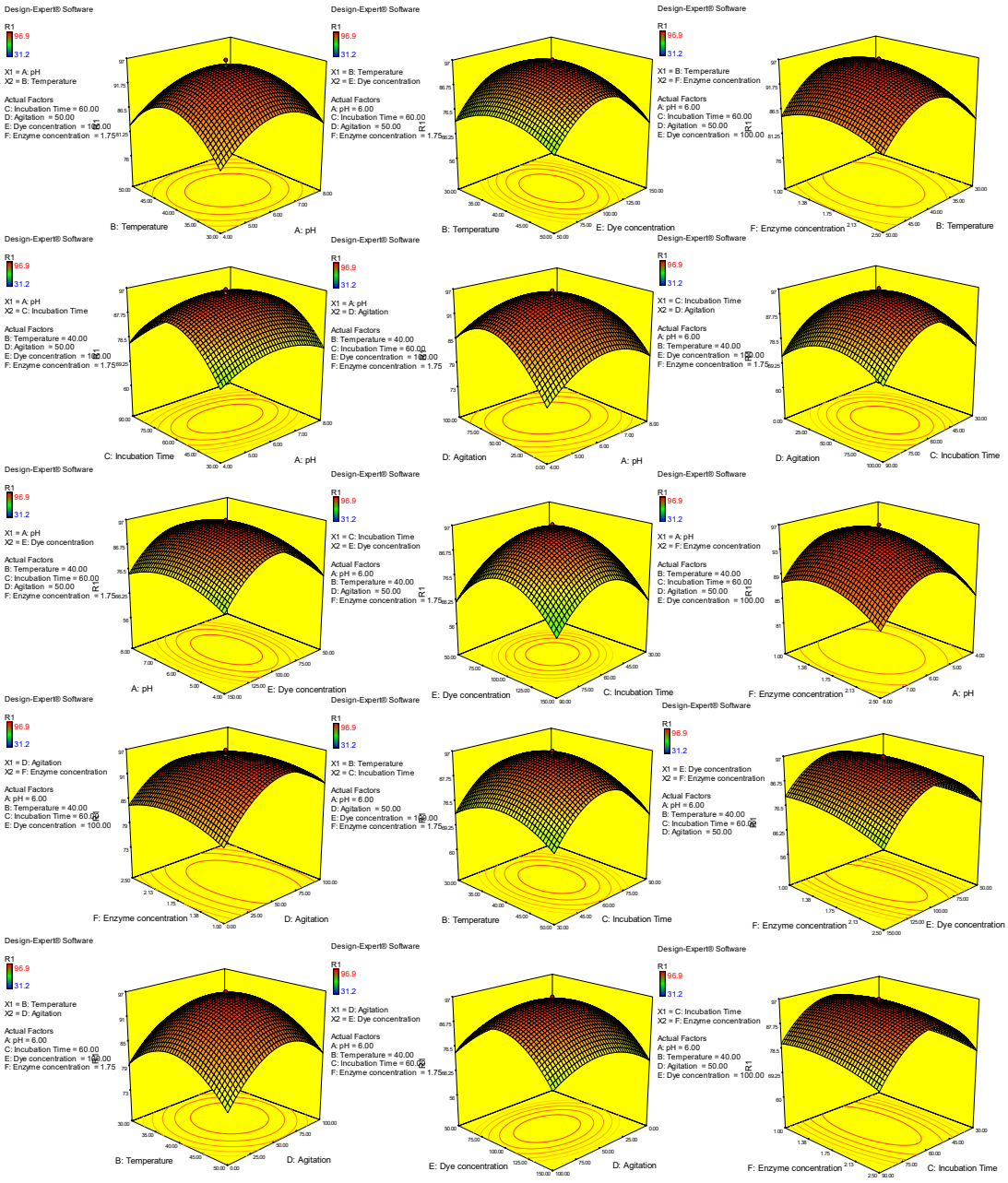

Figure 1. RSM Contour Plot and Surface Plot for Different Parameters (AB, AC, AD, AE, AF, BC, BD, BE, BF, CD, CE, CF, DE, DF, EF).

Table 2. Central Composite Design Matrix (CCD) and Their Observed Responses for Laccase-Assisted Decolorization of Remazol Brilliant Violet-5R

Run	pH	Temperature (°C)	Incubation Time (min)	Agitation (rpm)	Dye conc. (ppm)	Enzyme conc. (IU/mL)	Response	
							Actual	Predicted
1	8	50	90	0	150	1	40.3	40.79
2	8	50	90	100	50	2.5	38.9	38.86
3	4	50	30	100	150	1	46.2	46.47
4	4	50	30	0	50	1	31.8	31.80
5	8	30	30	0	150	1	46.9	47.18
6	8	50	90	100	150	2.5	32.5	32.47
7	8	30	30	100	150	2.5	40.1	40.14
8	6	40	60	50	100	1.75	96.9	95.72
9	4	50	90	100	50	1	49.5	49.20
10	4	50	30	100	50	1	46.2	46.03
11	6	40	60	50	100	1.75	95.3	95.72
12	6	40	60	50	100	1.75	95.7	95.72
13	4	30	30	0	150	2.5	34.2	34.90
14	4	30	90	0	150	2.5	37.2	37.10
15	8	50	90	0	50	2.5	41.2	41.10
16	8	50	90	0	50	1	35.3	35.43
17	8	30	30	100	50	2.5	42.8	42.65
18	8	50	30	0	50	2.5	43.7	43.82
19	4	30	30	0	50	1	31.2	31.24
20	6	40	60	50	178.25	1.75	58.4	57.50
21	4	50	90	0	50	2.5	43	42.71
22	4	50	90	100	50	2.5	47.6	47.65
23	4	30	30	100	50	2.5	42.7	42.49
24	2.9	40	60	50	100	1.75	81.2	81.00
25	6	40	106.95	50	100	1.75	60.2	60.42
26	6	40	13.05	50	100	1.75	60.4	59.59
27	4	50	30	100	50	2.5	48.7	48.77
28	4	30	30	100	150	1	47.8	47.71
29	8	30	30	0	150	2.5	44.6	44.46
30	8	30	90	100	50	2.5	37.2	37.31
31	8	30	30	100	50	1	42.1	42.37
32	4	30	90	0	50	2.5	39.2	39.13
33	4	50	90	100	150	2.5	38.6	39.03
34	6	40	60	128.25	100	1.75	79.1	78.82
35	4	50	90	0	150	1	40.9	40.81
36	6	40	60	50	100	1.75	94.9	95.72
37	4	30	90	0	150	1	44.9	44.75
38	8	50	90	100	150	1	43.1	42.97
39	4	50	90	100	150	1	50.2	50.17
40	8	50	30	100	50	2.5	44.1	44.28
41	6	40	60	50	100	1.75	95.2	95.72
42	8	30	30	100	150	1	49.2	49.45
43	8	30	90	100	50	1	41.4	41.33
44	8	50	90	0	150	2.5	36.9	36.88
45	9.1	40	60	50	100	1.75	81.5	81.10
46	4	30	90	100	150	2.5	37.4	37.25
47	8	50	30	100	150	1	43.5	43.58
48	6	40	60	50	100	1.75	95.6	95.72

Table 2. Continued

Run	pH	Temperature (°C)	Incubation Time (min)	Agitation (rpm)	Dye conc. (ppm)	Enzyme conc. (IU/mL)	Response	
							Actual	Predicted
49	6	24.35	60	50	100	1.75	78.7	78.40
50	4	50	30	0	50	2.5	41.1	41.13
51	4	50	30	100	150	2.5	39.4	39.62
52	4	30	90	0	50	1	37.1	37.20
53	4	30	90	100	50	1	45.5	46.11
54	8	30	90	0	50	2.5	41.9	42.16
55	4	30	30	100	50	1	42.9	42.86
56	8	30	90	100	150	2.5	35.2	35.33
57	8	30	90	0	150	1	49.2	49.37
58	6	40	60	-28.25	100	1.75	73.5	73.18
59	6	55.65	60	50	100	1.75	76.9	76.60
60	4	30	30	0	50	2.5	37.5	37.46
61	8	30	30	0	50	2.5	44.5	44.79
62	6	40	60	50	100	2.92	87.5	86.98
63	6	40	60	50	21.75	1.75	56.7	57.00
64	8	30	30	0	50	1	38.4	37.93
65	8	50	30	0	150	2.5	39.2	39.07
66	6	40	60	50	100	0.58	90.4	90.33
67	6	40	60	50	100	1.75	95.5	95.72
68	8	50	30	100	150	2.5	37.3	37.36
69	8	50	30	100	50	1	40.9	40.91
70	4	50	90	0	50	1	37.5	37.67
71	4	50	30	0	150	1	34.5	34.42
72	8	50	30	0	50	1	33.5	33.86
73	8	30	90	100	150	1	48.9	48.93
74	4	30	30	0	150	1	38.2	38.27
75	8	30	90	0	150	2.5	42.3	42.35
76	8	50	30	0	150	1	38.9	38.70
77	6	40	60	50	100	1.75	94.9	95.72
78	8	50	90	100	50	1	40.1	39.78
79	4	50	30	0	150	2.5	34.2	34.15
80	8	30	90	0	50	1	40.2	39.59
81	6	40	60	50	100	1.75	95.5	95.72
82	4	30	90	100	50	2.5	41.6	41.46
83	4	50	90	0	150	2.5	36.2	36.26
84	4	30	30	100	150	2.5	37.8	37.76
85	4	30	90	100	150	1	51.7	51.49
86	6	40	60	50	100	1.75	95.6	95.72

the adjusted R^2 value. The R^2 value of the enzyme activity was 0.995 that ensures the reasonable adjustment of the quadratic model to the experimental model (Table 3).

Response = 95.722 + 0.033A- 0.575 B + 0.265C + 1.802 D + 0.157 E - 1.070 F - 1.159 AB -1.075 AC - 1.793 AD + 0.5562AE + 0.159 AF -0.021 BC + 0.653 BD -1.103 BE + 0.775 BF -0.675 CD + 0.131 CE -1.0718 CF - 0.543 DE -1.647 DF -2.3968 EF -5.989 A^2 -7.4388B^2- 14.5832 C^2 -8.0512 D^2 -15.706 E^2 - 2.886 F^2

Validation of Model

In order to determine the correctness of the model, three verification experiments were performed using the statistically optimal requirements. The results showed a maximum decolorization efficiency of 95.7%, which is about 94.2% of the predicted value, implying a strong similarity between experimental and predicted values calculated from the model that confirms the precision and validity of the model.[29]

Table 3. Analysis of Variance

Source	Sum of Squares	*df*	Mean Square	*F* Value	*P* Value Prob > F
Model	35303.96	27	1307.554	8319.461	< 0.0001
A- pH	0.074758	1	0.074758	0.475656	0.4931
B- Temperature	22.78	1	22.78	144.9403	< 0.0001
C- Incubation Time	4.853682	1	4.853682	30.8821	< 0.0001
D- Agitation	223.7597	1	223.7597	1423.697	< 0.0001
E- Dye concentration	1.711979	1	1.711979	10.89266	0.0017
F- Enzyme concentration	78.91848	1	78.91848	502.1278	< 0.0001
AB	86.02562	1	86.02562	547.3478	< 0.0001
AC	73.96	1	73.96	470.5789	< 0.0001
AD	205.9225	1	205.9225	1310.205	< 0.0001
AE	19.8025	1	19.8025	125.9957	< 0.0001
AF	1.625625	1	1.625625	10.34322	0.0021
BC	0.030625	1	0.030625	0.194855	0.6605
BD	27.30062	1	27.30062	173.7033	< 0.0001
BE	77.88062	1	77.88062	495.5243	< 0.0001
BF	38.44	1	38.44	244.5789	< 0.0001
CD	29.16	1	29.16	185.5338	< 0.0001
CE	1.1025	1	1.1025	7.014781	0.0104
CF	73.53062	1	73.53062	467.8469	< 0.0001
DE	18.9225	1	18.9225	120.3966	< 0.0001
DF	173.5806	1	173.5806	1104.426	< 0.0001
EF	367.6806	1	367.6806	2339.41	< 0.0001
A^2	498.1552	1	498.1552	3169.569	< 0.0001
B^2	768.3951	1	768.3951	4889.001	< 0.0001
C^2	2953.093	1	2953.093	18789.39	< 0.0001
D^2	900.1118	1	900.1118	5727.063	< 0.0001
E^2	3425.28	1	3425.28	21793.73	< 0.0001
F^2	115.7271	1	115.7271	736.3266	< 0.0001
Residual	9.115751	58	0.157168		
Lack of Fit	6.246751	49	0.127485	0.399917	0.9807
Pure Error	2.869	9	0.318778		
Cor Total	35313.08	85			

Standard deviation =0.396444; Mean=51.93488; C.V. % =0.763349; PRESS=20.45463; R-Squared= 0.99742; Adjusted R-Squared=0.99622; Predicted R-Squared= 0.99421; Adeq Precision=285.0496.

Dye Toxicity

The obtained results of toxicity evaluation of untreated and laccase-treated dye solution showed that when Remazol Brilliant Violet 5R dye was used, the calculated growth inhibition (%) in the presence *S. aureus* MTCC 740, and *B. subtilis* MTCC 441 and *E. coli* MTCC 443, *P. aeruginosa* MTCC 741, and *S. typhi* MTCC 312 was found to be 72 ± 2.9, 68.5±3.5, 68.9 ± 6.1, 52.6 ± 4.3 and 62.8 ± 6.3 respectively. However, the growth inhibition (%) for laccase treated dye solution was 35 ± 1.2, 28.5±1.7, 29.9 ± 0.9, 32.5 ± 2.1 and 39.1 ± 1.3 respectively, that shows an effective reduction of toxicity.[30]

Enzyme Kinetics

The linear association among the initial velocity and dye concentration indicated that the decolorization is a first order reaction. Based on the MM and LB plots for enzymatic decolorization of Remazol Brilliant Violet 5R showed that V_{max} was 387 and the K_m value was 0.801. The Lineweaver Burk plot and Hans plot were also established for the reactions with the same values. The parameter space plot suggests the possible condition of reactions (Figure 2).

FT-IR Analysis

The efficacy of the degradation was studied using FT-IR. The peaks present in the graph got deteriorated after the dye was subjected to decolourisation. The FT-IR spectra of the adsorbents display the number of adsorption peaks, indicating the complex nature of the studied adsorbents. In FT-IR analysis, several peaks were observed in the spectra

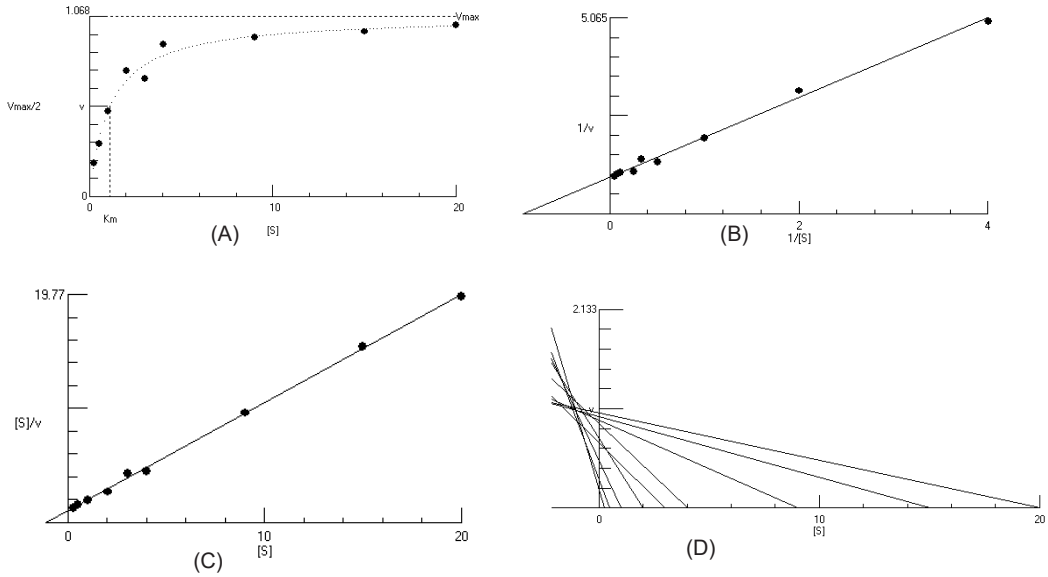

Figure 2. Kinetics study. (A) Michaelis-Menten plot. (B) Lineweaver-Burk plot.(C) Hans Plot. (D) Parameter space plot.

which indicated that the adsorbent is composed of various functional groups which are responsible for binding the chitosan. The bands 2083.2 and 2090.5 shows the variation in nitrile group (– C=N), the peaks 1640.8 and 1635.8 shows the variation in alkene groups (C=C) and the peaks 676 and 684.2 shows the variation in carbonyl group (C=O).[32-34]

There was significant variation in the peak before and after adsorption, which meaning that the high peak bond is stronger, so adsorption is more comparatively than the lower one (Figure 3). The deformation of OH and NH groups at the peaks 3700–3000 cm^{-1}.

Conclusions

The laccase enzyme production by *P. ostreatus* PKN04 was successfully optimized through the application of RSM-CCD to achieve the maximum favorable cultural conditions for laccase based decolorization process. Quadratic model was formed using 6 factors in terms of pH, temperature, incubation time, agitation, dye concentration, and enzyme concentration to epitomize the activity of laccase. The coefficients of independent variables were calculated using Design Expert 7.0. Decolorization of Remazol Brilliant Violet 5R was maximally 95.72%, which had occurred at 6.0 pH, 40°C temperature, 60 minutes incubation time, and 50 rpm agitation.

Authors' Contributions

TVP and GN developed the theory and performed the computations. TVP verified the analytical methods and AKK supervised the findings of this study. All authors discussed the results and equally contributed to the final manuscript.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

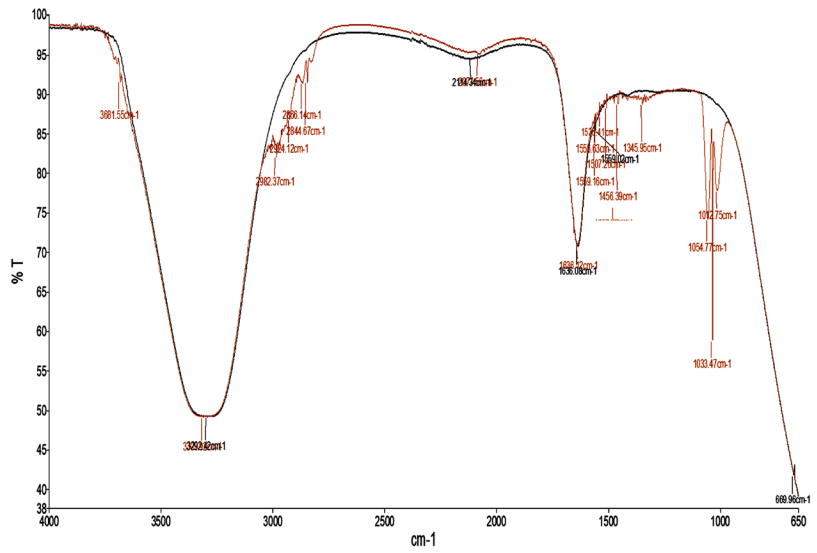

Figure 3. FT-IR Before and After.

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