Study the optimization of textile dyes biodegradation and lignin peroxidase production potential of locally isolated *Pleurotus ostreatus*, a white rot fungus

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Abstract

Textile industries are the major source of environmental pollution in the world because they release dyes, chemical and pigments during dying process in waste water. The current study was designed with aims of screening *Pleurotus ostreatus* biodegradation potential for four selected textile dyes i.e. direct pink B, direct yellow BG, dispersed red S3B and disperse yellow SRLB. The process was further optimized at two stages with Response Surface Methodology (RSM) under Central Composite Design (CCD). The effect of lignin peroxidase (LiP) on the biodegradation was also monitored and it was partially purified as well as characterized. The screening experiments showed that this fungus has higher biodegradation potential for disperse dyes (red S3B 75.25% & yellow SRLB 68.9% as compared to direct dyes (pink B= 33.53% & yellow BG= 32.1%). The biodegradation of disperse dyes was optimized by studying effect of growth parameters and nutritional parameters. The biodegradation of red S3B increased to 93.1% while biodegradation of yellow SRLB increased to 89.37% after optimization. Study of lignin peroxidase (LiP) confirmed its active role in biodegradation with its observed activities 89.33 IU/mL/min and 88.54 IU/mL/min during biodegradation of disperse red S3B & yellow SRLB, respectively. It was partially purified in the presence of 60 % ammonium sulfate, having optimum pH= 4.5 and temperature 30 ^{0}C. The LiP of *P. ostreatus* is quite active (Vmax= 277.7 mM/mL/min) ligninolytic enzyme and has good affinity for Azure B (Km=0.328 µM).

Key Words: *Pleurotus ostreatus*, dyes biodegradation, response surface methodology, lignin peroxidase

INTRODUCTION

Synthetic dyes are being widely used in so many industries like food, fabric and drug industries etc. Around 10, 000 different pigments and dyes are being used on commercial level (Ghascmi et al., 2010). Azo dyes contain double bond between nitrogen atoms and also contain sulfonic-groups due to which these are very difficult to degrade. As these dyes are coloring agents, they give colour to the water bodies thus lead to the risk for the overall environment.

Harmful and toxic substances in wastewater effluent cause many problems like aesthetic problems, skin irritation, skin sensitization, cancer, kidney diseases, other serious skin infections and even few concentrations of dyes danger for water bodies and aquatic life. Because of environmental legislation (Yaseen and Scholz, 2019) is necessary to breakdown the harmful and toxic substances before release into water as effluents (Zhao and Hardin,. 2007) Different fibers are dyed with different dyes. Degradation of dyes is relatively low. So, the need of white rot fungus for degradation of dyes and wastewater effluents cannot fulfill for industries and biotechnology point of view. Therefore, great attention required for enhancement the production of Increase in white rot fungi.

Demand of pure and good quality water is constantly increased with increase in global population. But at the same time water is being polluted with many toxic agents like dyes on daily basis, because it is also utilized by many industries for their processes. Dyes act as a persistant environmental pollutants, because they are toxic, mutagenic and carcinogenic in nature (Ismail et al., 2019).

Different type of physical methods and chemical methods used to remove dyes present in wastewater But there are some problems related to using these waste water treatment method including, economically less favourable, formation of concentrated sludge and its disposal, formation of some toxic products due to the use of chemicals (Adane et al., 2021) Biological methods are economically more effective and environment friendly. In these methods different microorganisms have been used for the biodegradation of dyes. Microorganisms degrade the pollutants by the action of their different intracellular or extracellular enzymes (Al prol, 2019; Adane et al., 2021).

White rot fungi are basidiomycetes and one of the type of filamentous fungi with the ability to degrade complex polymeric substances like lignin (Zuleta-correa et al, 2016). *Pleurotus ostreatus* is used as model organism in this study. It is considered as an efficient biodegrader of different organic compound including: lignin, synthetic dyes and other polycyclic aromatic hydrocarbons (PAHs). *Pleurotus ostreatus* is one of the most impotant medicinal mushrooms and the powered form of the fungus has been used to activate immune system (Archibald et al., 1997; Veena and pandey, 2012). Its biodegradation potential along with ligninolytic enzyme secretion potential was first time reported from Azad Kashimr Pakistan.

Materials and Methods

Chemicals and reagent

Malt extract, yeast extract, dextrose, peptone, agar, distilled water, fructose, sucrose and ammonium nitrate, sodium Tatarate buffer, Azure B, hydrogen peroxide, bovine serum albumen, Bradford reagent etc. were of analytical grade and purchase from local supplier.

Collection, isolation and maintenance of fungus

The white rot fungus *Pleurotus ostreatus* was provided by the Department of Biotechnology, MUST, Mirpur AJK. It is a macrofungus and their small pieces were used for isolation through culturing. The fungus was cultured on Yeast Malt Extract Agar (YMEA) medium of pH = 5.6 at 28 ^{0}C initially. Under aseptic conditions, the fungal spores were transferred on YMEA media plates. The plates were then incubated at 28 ^{0}C until full growth was obtained. (Tavares et al., 2005). Chemical composition of YMEA medium is given in the Table 1. Further, its broth i.e. YMEB was prepared for further use during biodegradation experiments (Table 2).

Table 1: Chemical composition of yeast malt extract agar (YMEA) media

Sr. No.	Chemicals	Quantity (g/L)
1.	Malt extract	2
2.	Yeast extract	0. 2
3.	Agar	1. 5
4.	d.H_2O	Up to 1000 mL

Table 2: Chemical composition of yeast malt extract broth (YME) media

Sr. No.	Chemicals	Quantity (g/L)
1.	Malt extract	2
2.	Yeast extract	0. 2
3.	d.H_2O	Up to 1000 mL

Collection of dyes

Four different synthetic textile dyes, commonly used in Pakistan including direct yellow BG (λmax= 395nm), direct pink B (λmax= 547nm), disperse yellow SRLB (λmax= 448nm) and disperse red S3B (λmax= 527nm) were used during the current study. All these dye were purchased from the local supplier and are available in the postgraduate laboratory, Department of Biotechnology, MUST Mirpur AJK.

Screening of selected dyes and biodegradation potential of *P.ostreatus*

For the screening experiments, 30 mL of 0. 02% (0. 006 g 30 mL) dyes solution, 5 mL YMEB media (pH = 5. 5) and 2mL fungal inoculum was mixed in a 100 mL autoclaved flask. The flask was then incubated at 28 ^{0}C for 120 hours (7 days). The absorbance of each sample was recorded initially and after every 24 hours at the λmax of each of 4 dyes. After taking absorbance, biodegradation percentage (B.D. %) was calculated by the following formula:

$$\text{B. D\%} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

Initial optimization of biodegradation by using RSM

For the optimization of biodegradation of the selected dye by *P.ostreatus*, Response Surface Methodology (RSM) was used to enhance the rate of dyes biodegradation. In the current study, CCD was applied to find out the optimized conditions under which there would be maximum biodegradation of the selected dye. (Gassara et al., 2011; Demirel and Kayan, 2012)

There were total 26 experiments designed by RSM under CCD with two central points as given in Table 3.

Table 1: Experiment design for the initial optimization of selected dyes

Exp. No.	pH	Temperature (^{0}C)	Dye Cone. (%)	Time period (hrs)
1.	6	20	0. 03	72
2.	9	40	0. 05	24
3.	9	20	0. 05	120
4.	3	40	0. 05	24
5.	9	40	0. 05	120
6.	6	30	0. 05	72
7.	3	20	0. 05	120
8.	9	20	0. 01	120
9.	3	20	0. 01	24
10.	9	40	0. 01	24
11.	6	30	0. 03	72
12.	6	30	0. 03	120

13.	9	40	0. 01	120
14.	3	40	0. 01	120
15.	9	20	0. 05	24
16.	6	30	0. 01	72
17.	9	30	0. 03	72
18.	3	20	0. 01	120
19.	6	30	0. 03	24
20.	6	30	0. 03	72
21.	3	20	0. 05	24
22.	9	20	0. 01	24
23.	6	40	0. 03	72
24.	3	40	0. 05	120
25.	3	30	0. 03	72
26.	3	40	0. 01	24

Effects of carbon and nitrogen sources

To check the effect of Carbon and Nitrogen sources on biodegradation of selected dyes by *P.ostreatus*. The biodegradation reaction mixture of dye was supplemented with different concentrations of two different Carbon sources i. e. Sucrose and Fructose and one Nitrogen source i. e. Ammonium nitrate (Mikiashvili et al., 2005). The JMP software designed sixteen (16) experiments for different concentrations ranging from 0. 1-0. 5 % of Sucrose, Fructose and Ammonium nitrate as given in Table 4.

Table 2: Experiment design to check the effect of Carbon and Nitrogen sources on selected dyes by *P.ostreatus*

Exp. No.	Fructose (%)	Sucrose (%)	Ammonium nitrate (%)
1.	0. 1	0. 5	0. 5
2.	0.5	0. 1	0. 5
3.	0. 3	0. 3	0. 1
4.	0. 3	0. 3	0. 3
5.	0. 5	0. 3	0. 3
6.	0. 1	0. 3	0. 3
7.	0. 1	0. 1	0. 5
8.	0. 5	0. 1	0. 1
9.	0. 3	0. 3	0. 3
10.	0. 3	0. 3	0. 5
11.	0. 5	0. 5	0. 5
12.	0. 5	0. 5	0. 1
13.	0. 3	0. 5	0. 3
14.	0. 1	0. 1	0. 1
15.	0. 3	0. 1	0. 3
16.	0. 1	0. 5	0. 1

Lignin peroxidase activity assay

The protocol used for LiP activity assay was described by Archibald, 1992 and Jovi et al. , 2018. LiP activity assay was performed by adding 1 mL of 100 mM Sodium tartarate buffer, 500 uL of 0. 16 mM Azure B and 500 uL of 0. 1 M H_2O_2 in a clean and dry test tube. Then 500

uL of biodegraded dye sample containing enzyme was added in it and absorbance was measured at 651 nm i. e. initial OD at to with the help of spectrophotometer. In control, distilled water was added instead of substrate. After 10 minutes, absorbance was measured at 651 nm i. e. final OD at t_{10} was taken. During 10 minutes, there was no color change and the absorbance was decreased after 10 minutes. Enzyme activity can be measure by the following formula:

$$A = €651 \times c \times 1 \quad (€651 = 85,000\ M^{-1}cm^{-1})$$

Where,

A = absorbance (t_0 __ to) c = concentration or enzyme activity

€651 = extinction coefficient 1 = path length

Partial purification of LiP

For partial purification, first of all the Biodegraded dye sample was centrifuged at 6000 RPM for 15 minutes. then the supernatant was used for the partial purification of dissolved LiP enzyme. Ammonium sulphate precipitation protocol proposed by Hyman Lab, Max Planck Institute of Molecular Cell Biology and Genetics was used in this study. The enzyme sample was saturated with different concentration of $(NH_4)_2SO_4$ ranging from 40 to 90 percent to obtain the maximum precipitation. The $(NH_4)_2SO_4$ weighed for different saturation levels (percentage) as described by Green and Hughes, 1955 and mentioned in table 5. It was added slowly and mixed into the enzyme sample to avoid clump formation. Then the mixture was placed at 4^0C to allow the precipitation of proteins with constant stirring or gentle shaking manually for 30-40 minutes. After that, the mixture was centrifuged at 6000 RPM for 20 minutes. The supernatant was collected in a separate tube and the pellet was dissolved in 2 ml autoclaved distilled water. Then the activity assays of LiP was performed with both supernatant and pellet for the comparison**.**

Table 3: Ammonium Sulfate required in grams for specific saturation%

Sr. No.	$(NH_4)_2SO_4$ Saturation%	Weight of $(NH_4)_2SO_4$ in grams
1.	40	1. 215
2.	50	1. 565
3.	60	1. 95
4.	70	2. 36
5.	80	2. 805
6	90	3. 31

Characterization of LiP

After partial purification, the pellet sample with maximum precipitated ligninolytic enzymes having highest activities was subjected to the characterization of different kinetic parameters of lignin peroxidase. These parameters include:

1. Determination of optimum temperature
2. Determination of optimum pH
3. Effect of substrate concentration to determine Km and Vmax

Determination of Optimum Temperature

For determination of optimum temperature of LiP, the activity assays were performed at following temperatures: 24 ^{0}C, 27 ^{0}C, 30 ^{0}C, 33 ^{0}C, 36 ^{0}C and 39 ^{0}C.

Determination of Optimum pH

Enzymes are also very sensitive to slight changes in pH and the specific pH at which the enzymes show maximum activity is called optimum pH. To determine the optimum pH of ligninolytic enzymes, For determination of optimum pH for maximum LiP sodium tatarate buffer buffers of five different buffers of different pH i. e. 3. 5, 4, 4. 5, 5 and 5. 5 were used for the activity assays. For the control of activity assays at different pH, the buffer of pH mentioned in standard protocol was used

Effect of Substrate Concentration to determine Km and Vmax

For the determination of K_m and V_{max} of LiP , the activity assay were performed with different substrate concentrations Azure B 0. 5 mM. 1 mM, 2mM, 4 mM, 8 mM and 16 mM. Then for the determination of Km and Vmax, a double reciprocal graph was plotted between 1/[S] and 1/V (where, [S] denotes substrate concentration and V denotes velocity or enzyme activity.

Results and Discussion

Pleurotus ostreatus was cultured on YMEA (pH-5.5) plate and incubated at 28°C. Full fungal growth was obtained within 4-5 days. Similarly, the fungal inoculum was prepared within 3-4 days when the number of fungal spores reached to 10^7-10^9 spores/mL, then it was stored at 4 °C before further use.

Screening of biodegradation of selected dyes by *P.ostreatus*

The biodegradation of both disperse dyes was higher than direct dyes by *P.ostreatus* (Fig. 1). The biodegradation of disperse Red S3B dye was 75.25%, disperse yellow was 68.9%, direct pink B & direct yellow 33.53%. Based on screening results the biodegradation of disperse dyes was further optimized by using Response Surface Methodology.

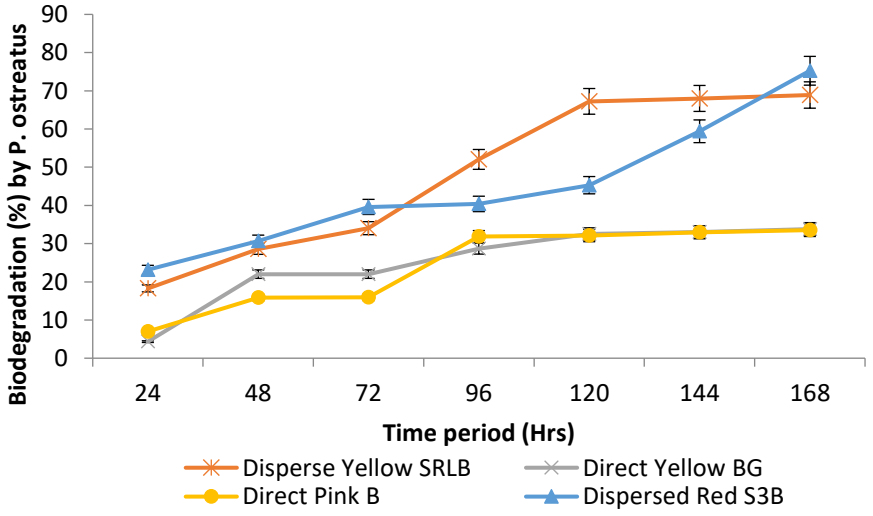


Figure 1: Screening of biodegradation potential of *P. ostreatusr* for selected textile dyes.

Optimization of growth parameters for the biodegradation of disperse dyes

On the basis of screening results, the biodegradation of direct dyes was optimized by using RSM under Central Composite Design (CCD). For this, effect of four different growth parameters i.e. pH, temperature, dye concentration and time period was checked on our desired response i.e. biodegradation.

Optimization of biodegradation of disperse yellow SRLB by *P.ostreatus*

After performing the experiments according to strategy given in table 3, there was 10% increase in the biodegradation. The biodegradation of disperse yellow SRLB was increased from 68.9% to 78.23 % at pH 5.5, temperature 28.3^{0}C, 0.05% dye and after 120 hrs (Fig. 2). Graphs of the each parameter indicating the variations in it leads to the reduced biodegradation (%) of the dye. The 3D response surface graphs showing the interaction between two parameters and their impact on biodegradation further confirms that biodegradation is maximum around suitable value of each parameter and moving either side leads to decrease in biodegradation (Fig. 3a, b, c). There is positive interaction among the parameters that gives better response at a point. The higher value of *F.ratio* and lower *P value* indicated that there is significant effect of the parameters under study on biodegradation (Fig.3d).

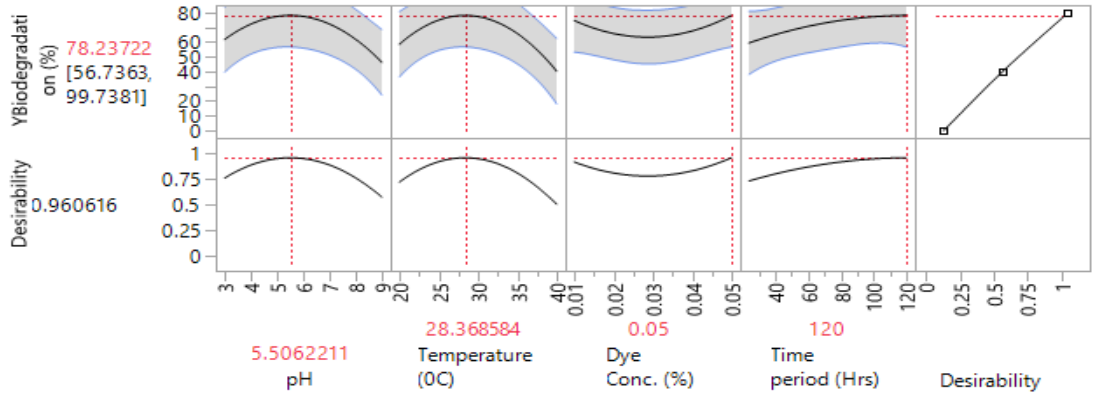
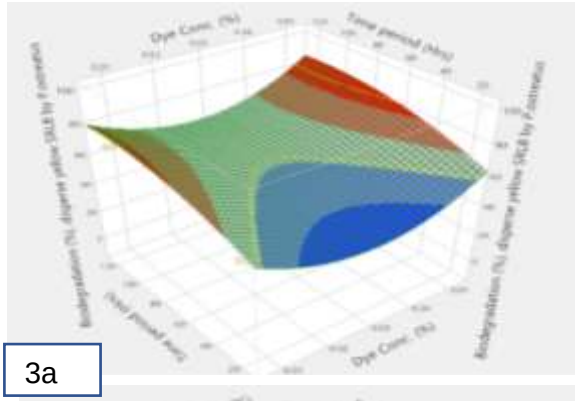
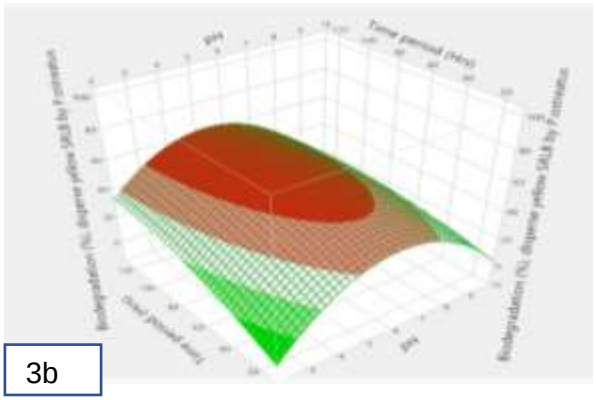


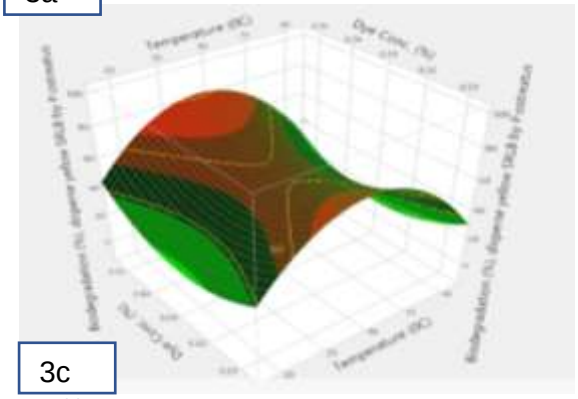
Figure: 2 Desirability plot obtained through JMP indicating the most suitable value of studied parameters and maximum biodegradation of disperse yellow SLRB by *P.ostreatus*



3a



3b



3c

ANOVA table

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	10	1489. 2920	148. 929	44. 0619
Pure Error	1	3. 3800	3. 380	Prob > F
Total Error	11	1492. 6720		0. 1168
				Max RSq
				0. 9998

3d

Figure 3: Response surface 3D graphs showing interaction between (a) dye concentration and time period (b) pH and time period (c) temperature and dye conc. (d) statistical analysis during the biodegradation of disperse yellow SRLB by *P. ostreatus*.

Optimization of biodegradation of disperse red S3B by *P.ostreatus*

Optimization experiments enhanced the biodegradation of disperse red S3B from 75.25 to 93.19%. This 18% increase in biodegradation was achieved at pH 5.5, temperature 29.3 ^{0}C, 0.05% dye conc. and after 120 hrs (Fig. 4). The effect of pH and temperature on biodegradation is more compared to other two parameters indicated by the parabolic and flat shape of the graphs of individual parameter.

The interaction between two parameters and their effect on the biodegradation of disperse red S3B can be analyzed from 3D response surface graphs (Fig. 5a, b, c). The maximum biodegradation of dye is due to combined effect of all the parameters that interact positively with each other. The peak point of the graphs indicated the area where maximum response obtained and any change in values of the parameters under study brings variation in the biodegradation. Higher value of *F.ratio* and lower *P value* (<0.035) indicated the significant effect of all the variable parameters on the biodegradation (Fig. 5d). It's essential to adjust the values at optimum level to achieve maximum biodegradation.

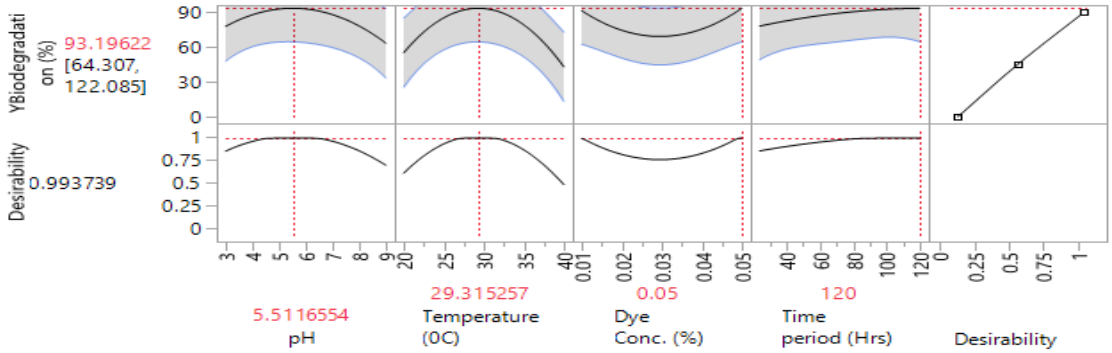
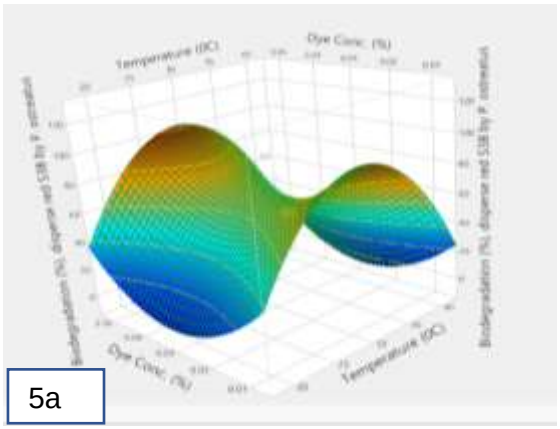
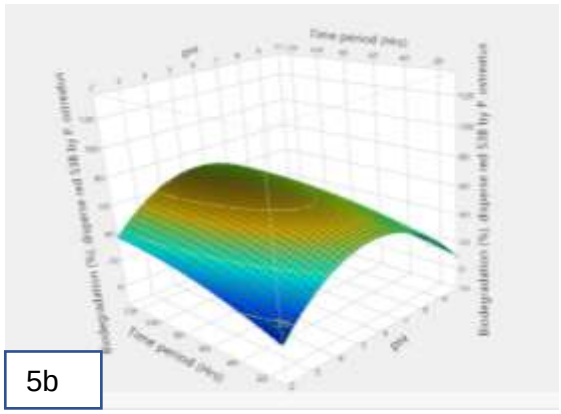


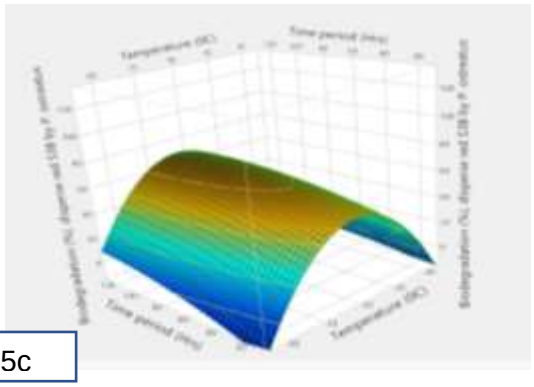
Figure: 4 Desirability plot obtained through JMP indicating the most suitable value of studied parameters and maximum biodegradation of disperse red S3B by *P.ostreatus*



5a



5b



5c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	10	2665. 6456	266. 565	492. 9078
Pure Error	1	0. 5408	0. 541	Prob > F
Total Error	11	2666. 1864		0. 0350*
				Max RSq
				1. 0000

5d

Figure 5: Response surface 3D graphs showing interaction between various parameters during the biodegradation of disperse red S3B *by P. ostreatus* (a) temperature and dye concentration (b) PH and time period (c) temperature and time period (d) statistical analysis of results showing significant effects of parameters.

Effect of carbon and nitrogen sources on biodegradation of disperse yellow SRLB

Availability of additional carbon and nitrogen sources in the media enhance the microbial growth, which may leads to higher biodegradation of coloring agents. In current study biodegradation of disperse yellow SRLB was enhanced from 78.25- 89.37 % (11%) when media provided with 0.1% fructose, 0.2% sucrose and 0.5% ammonium nitrate (Fig. 6). The 3D response surface graphs showing the positive interaction between these nutritional sources which leads to enhanced dye biodegradation (Fig. 7a-c). Any deviation from above mentioned amount has negative impact on the biodegradation. The significant effect of these sources was further confirmed by statistical analysis, indicating higher *F.ratio* and lower *P.value* (<0.11) (Fig. 6d).

Effect of carbon and nitrogen sources on biodegradation of disperse red S3B

The biodegradation of disperse red S3B dye was increased from 75.25 to 86.40% (11%) after the additional of readily available carbon and nitrogen sources. The most suitable concentration of each nutrient is 0.5% fructose, 0.1% sucrose and 0.2% ammonium nitrate for maximum biodegradation (Fig. 8). The graphs obtained after analysis of results through JMP software showed that increase in the biodegradation is due to positive interaction between these sources. The peak points indicated the maximum response at suitable conditions, any change in the concentration leads to decrease in biodegradation (Fig. 9 a-c). The higher *F.ratio* and lower

P.value statistically confirmed the significant impact of carbon and nitrogen sources on the biodegradation of direct yellow BG (Fig. 9d).

Study of Lignin peroxidase (LiP) during the optimization of biodegradation of disperse dyes by *P. ostreatus*

Studied have reported that dyes biodegradation by fungus is due to the action of ligninolytic enzymes secreted by it. To check the role of LiP in the biodegradation of direct dyes by *P.ostreatus* the activity assays were performed along with biodegradation. The production and activity of LiP enzyme may be affected by different parameters including pH, temperature, dye concentration and incubation period, the conditions we provided during the optimization of biodegradation process. So, it is important to study the conditions suitable for LiP production and its activities.

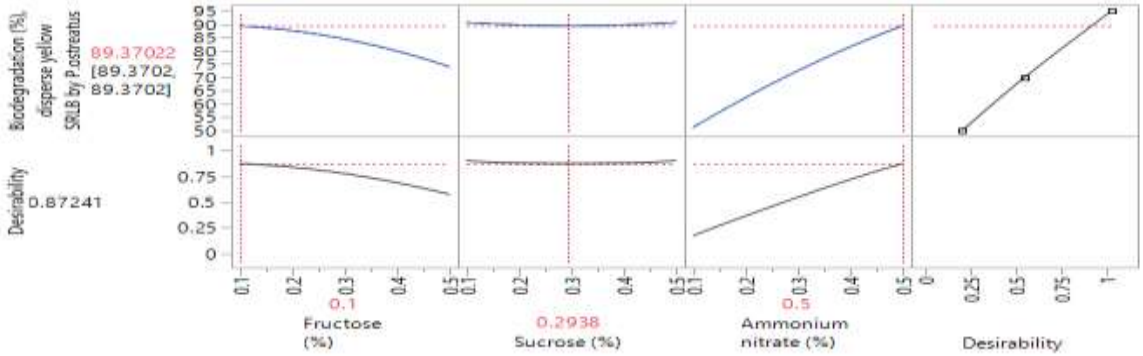
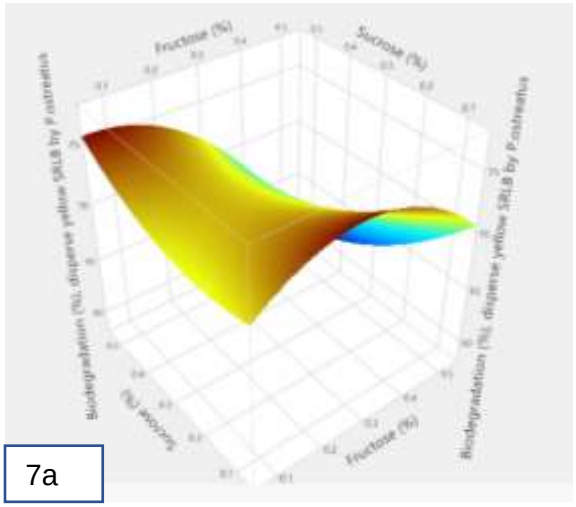
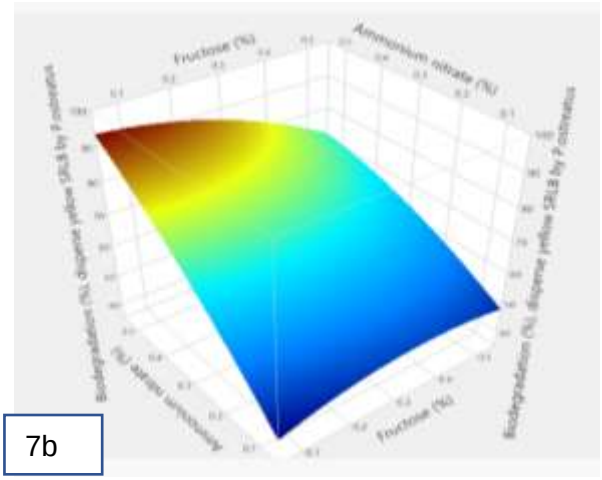


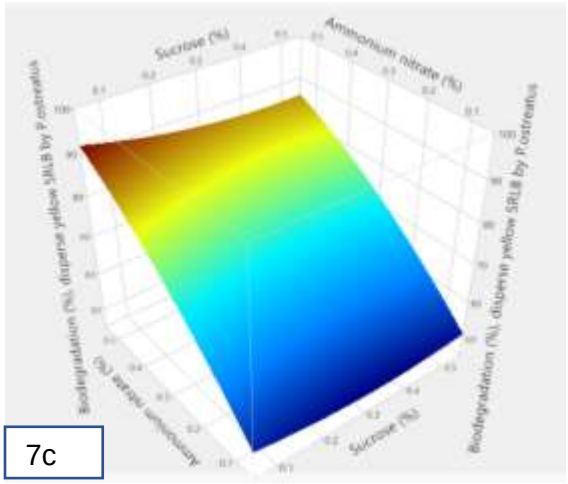
Figure: 6 Desirability plot obtained from JMP, showing the most suitable value of carbon and nitrogen sources for maximum biodegradation of disperse yellow SRLB by *P.ostreatus*



7a



7b



7c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	5	265.47931	53.0959	47.1963
Pure Error	1	1.12500	1.1250	Prob > F
Total Error	6	266.60431		0.1100
				Max RSq
				0.9996

7d

Figure 7 Response surface graphs showing interaction between (a) fructose and sucrose (b) fructose and ammonium nitrate (c) sucrose and ammonium nitrate (d) ANOVA table during biodegradation of disperse yellow SRLB by *P.ostreatus* in the presence of different carbon and nitrogen sources.

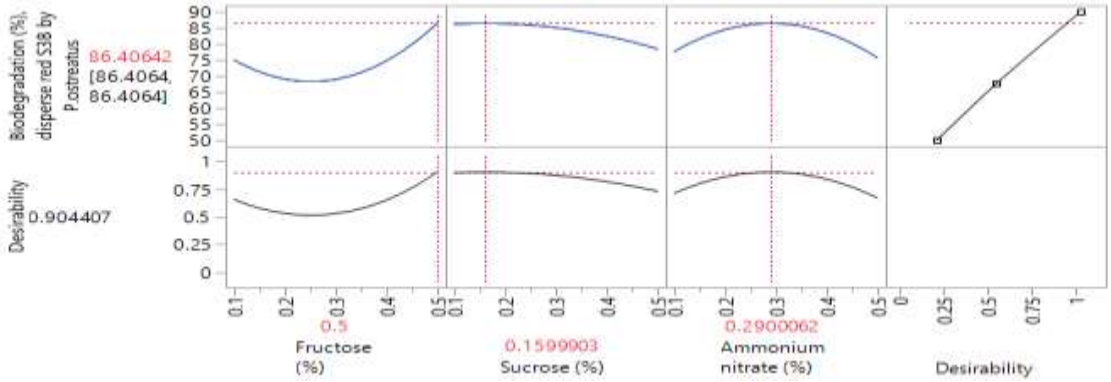
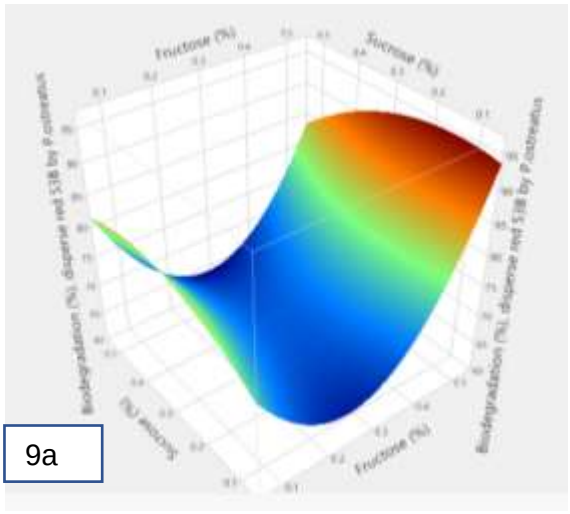
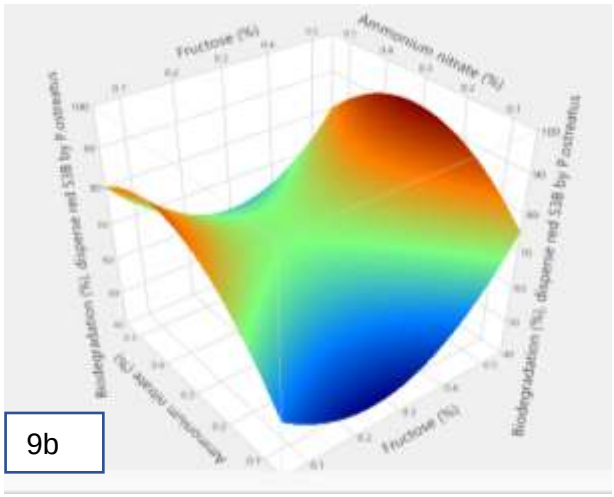


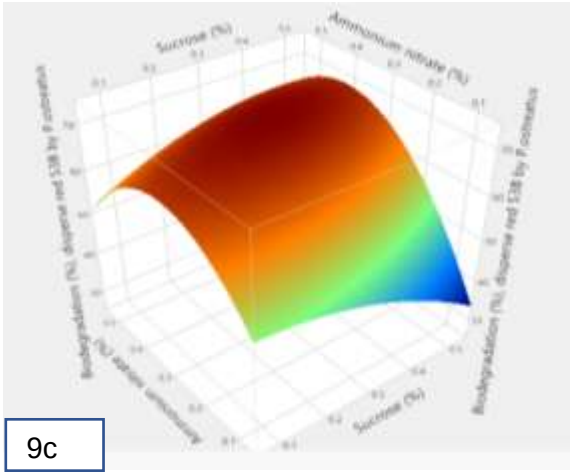
Figure: 8 Desirability plot obtained from JMP, showing the most suitable values of carbon and nitrogen sources for maximum biodegradation of disperse red S3B by *P.ostreatus*



9a



9b



9c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	5	937. 5828	187. 517	1. 6668
Pure Error	1	112. 5000	12. 500	Prob > F
Total Error	6	1050. 0828		0. 5264
				Max RSq
				0. 9523

9d

Figure 9 : Response surface graphs showing interaction between (a) fructose and sucrose (b) fructose and ammonium nitrate (c) sucrose and ammonium nitrate (d) ANOVA table during biodegradation of disperse red S3B by *P.ostreatus* in the presence of different carbon and nitrogen sources.

LiP activity during optimization of biodegradation of disperse red S3B dye

There was 89.33 IU/mL/min activity of LiP during the optimization of biodegradation at pH 5.5, 29.32 ^{0}C, 0.05% dye conc. and 120 hrs time period (Fig.10). The conditions for maximum enzyme activity were similar to the conditions for maximum biodegradation, which confirmed the role of LiP in the biodegradation process. The 3D response surface graphs showing the interaction between two parameters during the biodegradation and relative LiP activity during the process (Fig. 11a-c). There is positive interaction between these parameters which is responsible for higher LiP activities that leads to biodegradation of disperse red S3B, as reported in Fig. 3. Higher *F.ratio* and lower *P value*, statistically confirm the significant effect of studied parameters on enzyme activity (Fig. 11d).

LiP activity during optimization of biodegradation of disperse yellow SRLB

The activity of LiP was 73.51 IU/mL/min at 5.38 pH, 28.4 ^{0}C, 0.01% dye conc. and after 117 hrs during the biodegradation of disperse yellow SRLB (Fig.12). These conditions are closer to that for maximum biodegradation (Fig. 4), confirming the role of LiP enzyme in the biodegradation of

disperse yellow SRLB by *P.*ostreatus Similarly, the parameters under study interact positively with higher LiP activities at similar conditions where higher rate of biodegradation observed (Fig. 13a-c). The effect of all the parameters studied, on the LiP activity was significant as indicated by the higher *F.ratio* and lower *P value* during statistical analysis (Fig. 13d).

LiP activity during the biodegradation of disperse yellow SRLB in the presence of carbon and nitrogen sources

The increase in biodegradation of disperse yellow SRLB dye was observed after the addition of fructose & sucrose as additional carbon sources and ammonium nitrate as nitrogen source, similar shift was observed in LiP activity. The activity was 88.54 IU/mL/min in the presence of 0.1% fructose, 0.5% sucrose and 0.5% ammonium nitrate (Fig. 14), indicating the its role in increasing in biodegradation. There is synchronizing interaction between these sources which leads to higher activities at similar conditions where rate of biodegradation increases (Fig. 15a-c). The statistical analysis further confirm the significant effect of carbon and nitrogen sources on LiP activity indicated by higher *F.ratio* and lower *P. value* (Fig. 15d).

LiP activity during the biodegradation of disperse red S3B in the presence of carbon and nitrogen sources

There was increased in the LiP activity was obtained during the biodegradation of disperse red S3B i.e. from 73.51 to 83.3 IU/mL/min in the presence of 0.5% fructose, 0.3% sucrose and 0.31% ammonium nitrate (Fig. 16). There was small increase in biodegradation of dye during this step because higher biodegradation and LiP activity was achieved during first step of optimization of biodegradation. The 3D response surface graphs indicated the positive interaction between carbon and nitrogen sources for increase in LiP activity (Fig. 17a-c). Moreover, the conditions for higher LiP activity are similar to those of higher biodegradation (%), indicated the significant role of this enzyme in biodegradation of disperse red S3B. The higher *F.ratio* and lower *P. value*, shows the results are significant statistically (Fig. 17d).

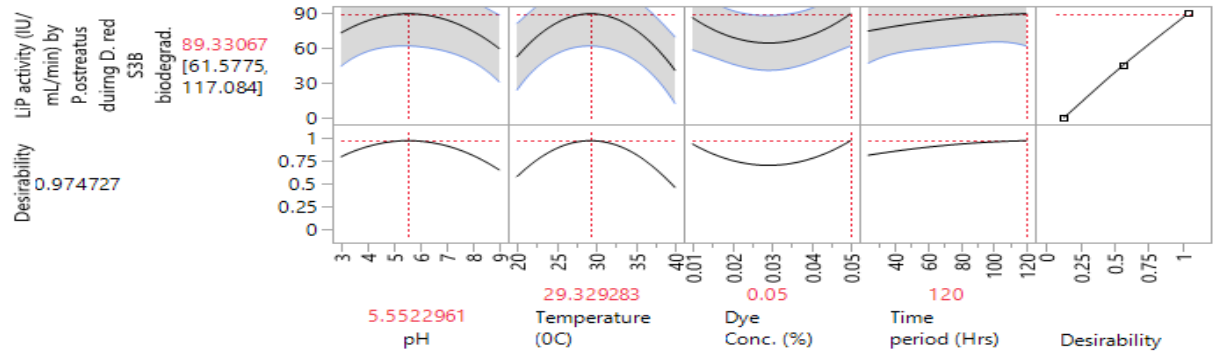
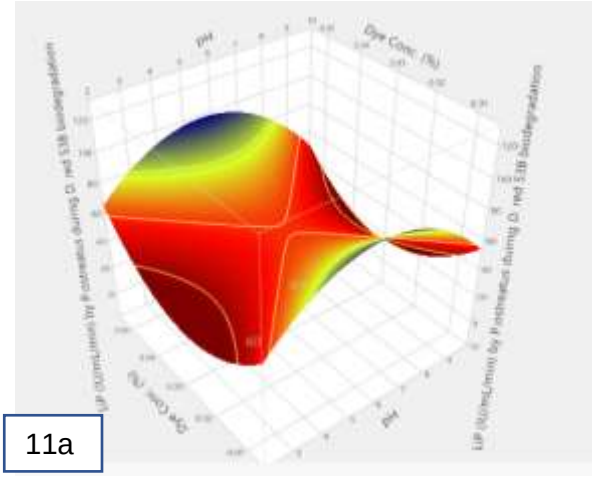
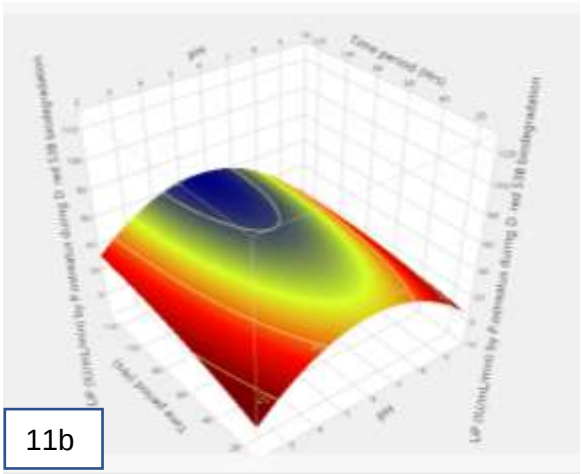


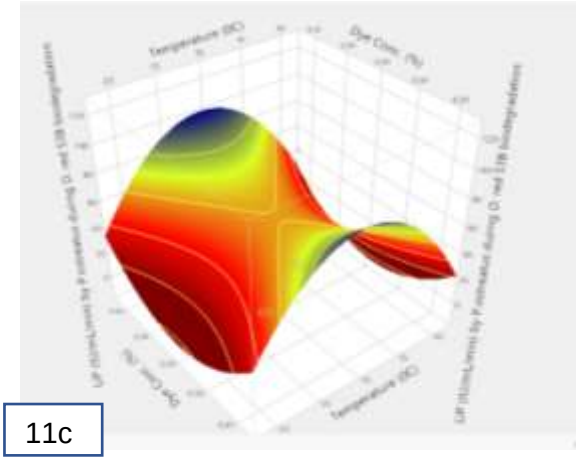
Figure: 10 Desirability plot obtained from JMP, showing the most suitable value of studied parameters for higher LiP activity during optimization of biodegradation of disperse red S3B by *P.ostreatus*



11a



11b



11c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	14566. 795	1040. 49	4. 6608
Error	11	2455. 643	223. 24	Prob > F
C. Total	25	17022. 438		0. 0072*

11d

Figure 11: Response surface graphs showing interactive effect of (a) pH and dye concentration (b) pH and time period on LiP activity (c) temperature and dye conc. (d) statistical analysis during the optimization of biodegradation of disperse red S3B by *P. ostreatus*.

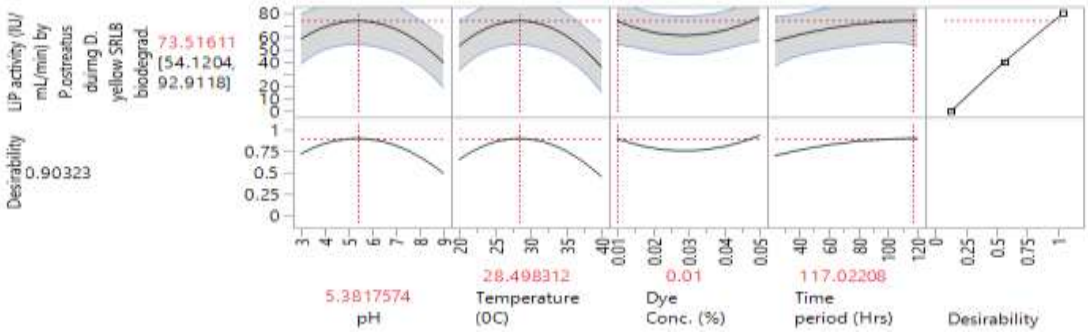
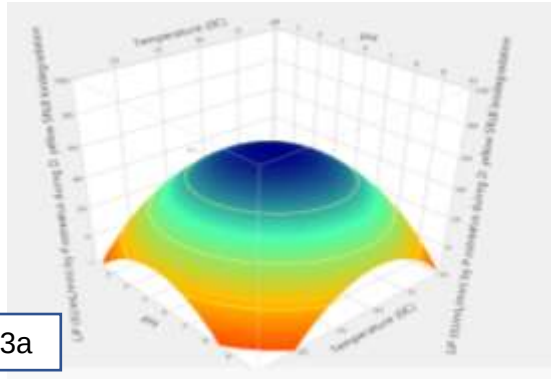
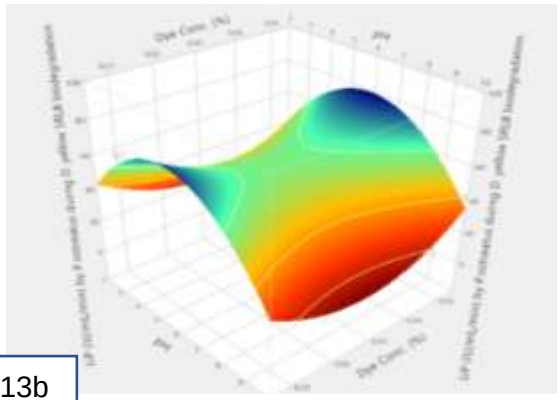


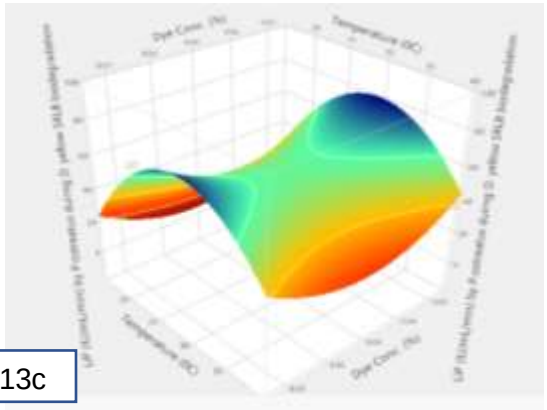
Figure:12 Desirability plot showing the most suitable value of studied parameters for higher LiP activity during optimization of biodegradation of disperse red S3B by *P.ostreatus*



13a



13b



13c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	11945. 583	853. 256	6. 9781
Error	11	1345. 042	122. 277	Prob > F
C. Total	25	13290. 625		0. 0013*

13d

Figure: 13 Response surface graphs showing interactive effect of (a) PH and temperature (b) PH and dye concentration (c) temperature and dye concentration (d) ANOVA table on LiP activity during the optimization of biodegradation of disperse red S3B by *P. ostreatus.*

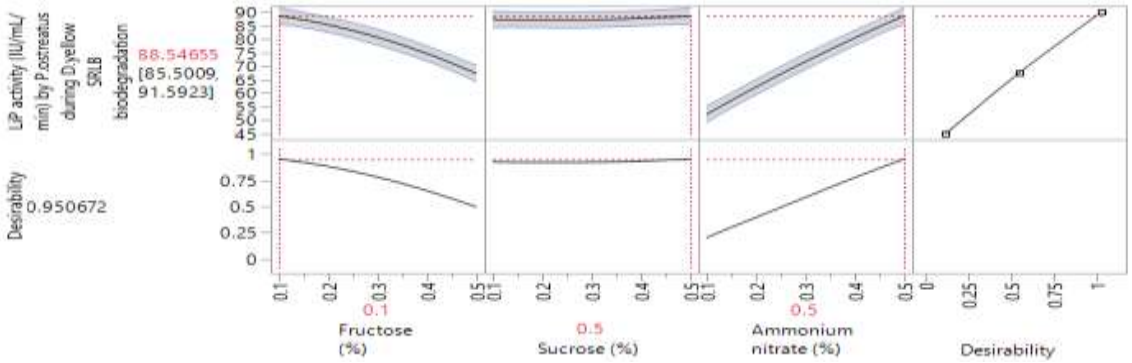
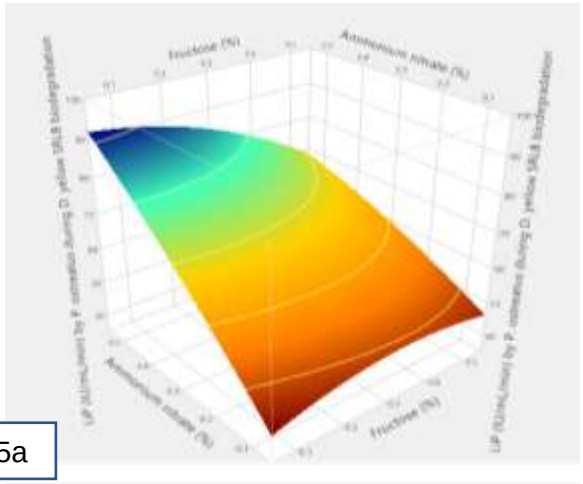
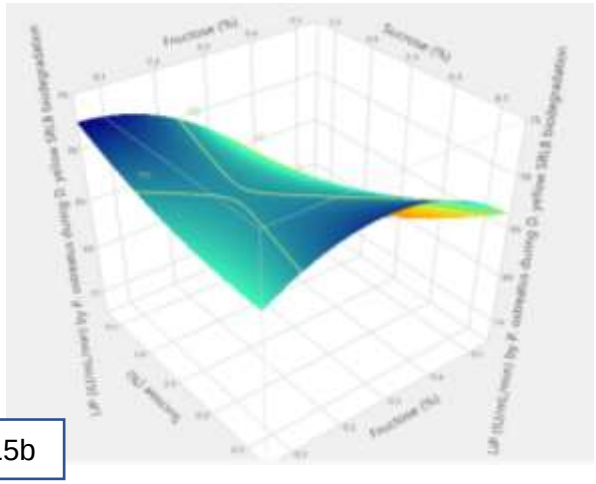


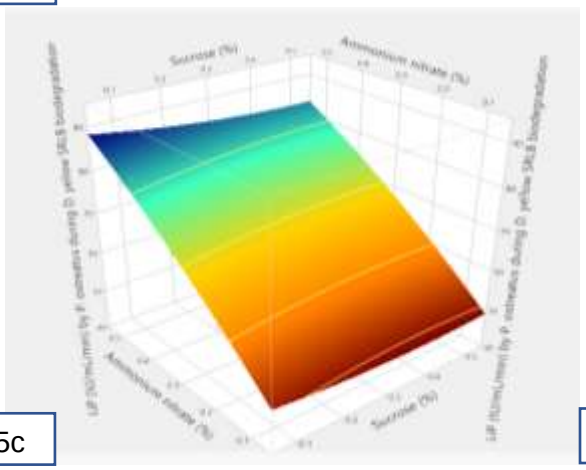
Figure: 14 Desirability plot obtained from JMP, showing the most suitable value of carbon and nitrogen sources with LiP activity during the biodegradation of disperse yellow SRLB by *P.ostreatus*



15a



15b



15c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	2480. 0672	275. 563	141. 5229
Error	6	11. 6828	1. 947	Prob > F
C. Total	15	2491. 7500		<. 0001*

15d

Figure: 15 Response Surface graphs showing interactive effect of (a) Fructose and Ammonium nitrate (b) Fructose and Sucrose, (c) Sucrose and Ammonium nitrate (d) statistical

analysis on lignin peroxidase of *P. ostreatus* in the presence of different carbon and nitrogen sources.

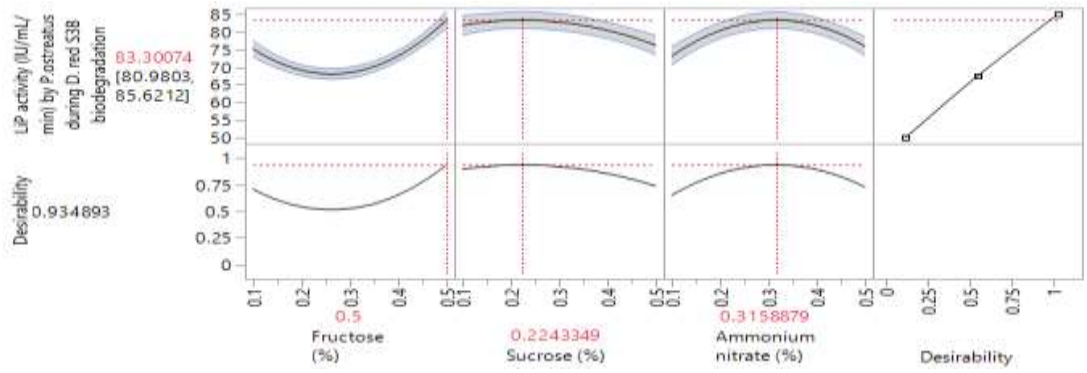
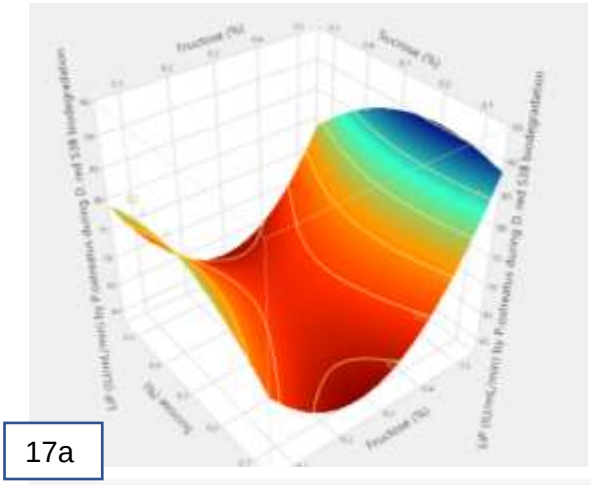
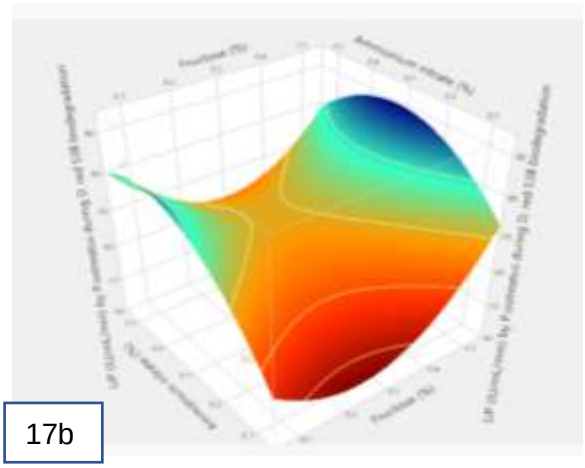
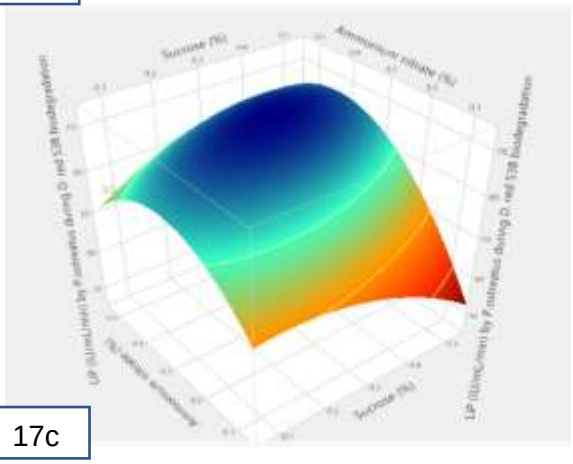


Figure: 16 Desirability plot showing the most suitable value of carbon and nitrogen sources with LiP activity during the biodegradation of disperse red S3B by *P.ostreatus*


17a


17b


17c

ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	1245. 3677	138. 374	78. 5486
Error	6	10. 5698	1. 762	Prob > F
C. Total	15	1255. 9375		<. 0001*

5d

Figure: 17 Response Surface graphs showing interactive effect of (a) Fructose and Sucrose (b) Fructose and Ammonium nitrate (c) Sucrose and Ammonium nitrate (d) statistical analysis on lignin peroxidase of *P. ostreatus* in the presence of different carbon and nitrogen sources.

Partial Purification of LiP by Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed for partial purification of LiP in the biodegraded samples. The results showed that there was maximum precipitation of LiP in the presence of 70% of $(NH4)_2SO_4$. There was highest activity at 53.5 IU/mL/min obtained with decline on either side, indicating the lowering of protein concentration (Fig. 18).

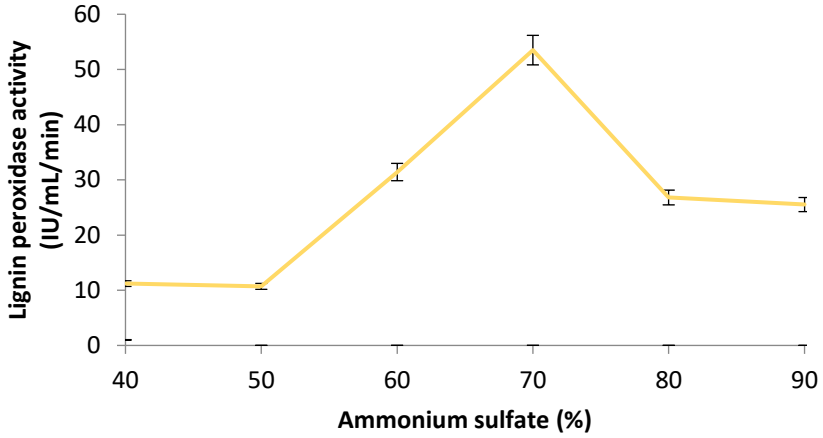


Figure 18: Ammonium sulfate precipitation of LiP at different saturation level

Characterization of LiP

The LiP was further subjected to the characterization for the determination of optimum temperature, pH and finding values of Km and Vmax.

Determination of optimum temperature

For the determination of optimum temperature, LiP was incubated along with its substrate at different temperature for 10 minutes and then absorbance was recorded. The results are given in the Fig. 19, showed that maximum activity was obtained at 30 ^{0}C i.e. 33.6 IU/mL/min. The activity reduces to 0.58 IU/ml/min at 39^{0}C, indicating the maximum denaturation of enzyme.

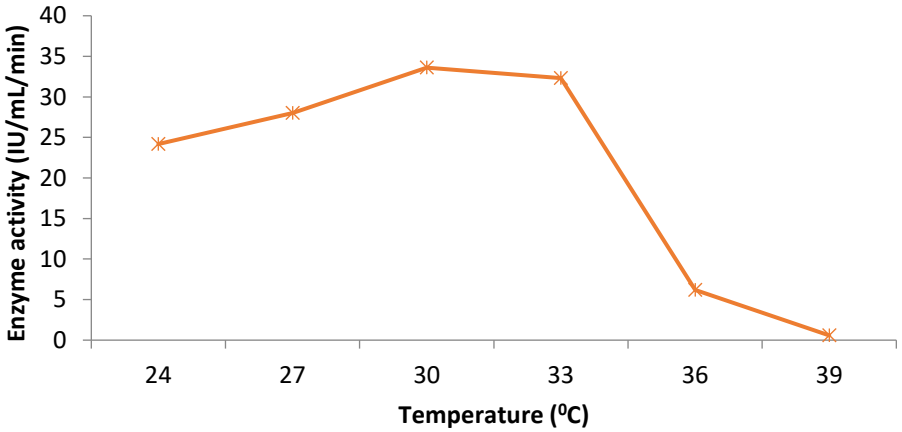


Figure 29: Effect of temperature on lignin peroxidase activity

Determination of optimum pH

Enzymes are very sensitive to slight change in pH, buffers are usually used to maintain the pH of the surrounding environment and the pH should be maintained for the proper functioning. The results showed that it has optimum pH in the acidic range i.e. pH 4 with activity 48 IU/mL/min (Fig. 20). There was quite high activity between pH 3 to 4.5 and maximum denaturation observed at pH 5.

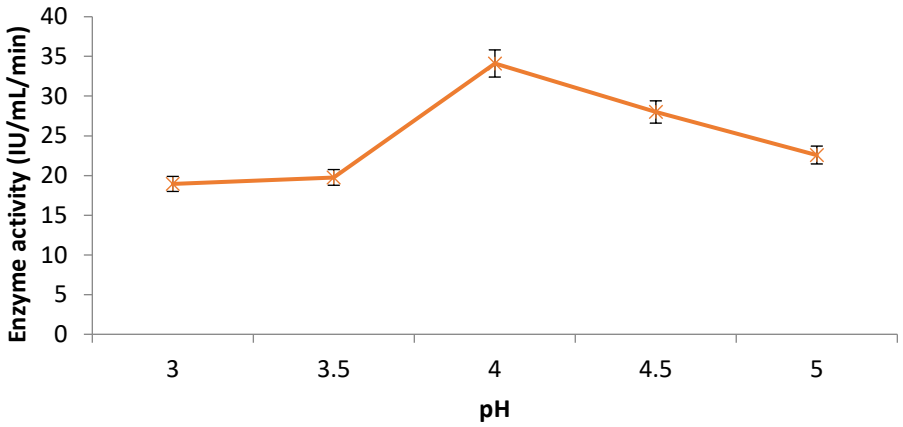


Figure 20: showed optimum pH of Lignin peroxides

Effect of Substrate Concentration to Determine Km and Vmax

For determining the effect of substrate concentration on LiP, activity assays were performed with different substrate concentrations. Values were used to draw Lineweaver-Burk plot between 1/[S] and I/V_0 and to calculate Km & Vmax of LiP (Fig. 21). The LiP of *P.ostreatus* has Vmax 277.7 mM/ml/min and Km 0.328 µM, indicating it is an active enzyme with good affinity towards its substrate.

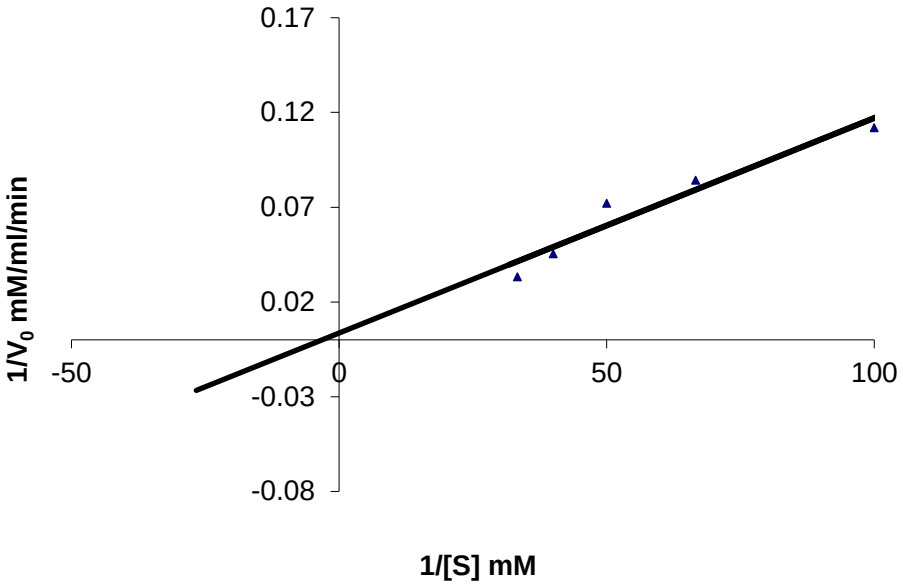


Figure 21: Lineweaver-Burk plot for the determination of Km and Vmax of LiP

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