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Research Article

EFFECTIVE DECOLORIZATION OF TEXTILE EFFLUENT BY LACCASE (LCC2) FROM *PLEUROTUS OSTREATUS* IMI 395545 USING ORTHOGONAL ARRAY MODEL OF TAGUCHI DOE METHODOLOGY.

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ABSTRACT

Taguchi designing of experiments (DOE) methodology was applied to optimise the decolorization of real textile effluent samples were collected at the point of effluent drainage pipe outlet in Erode Cauvery river near the new bridge, Tamil Nadu, India. Purified laccase LCC2 from Pleurotus ostreatus IMI 395545 was used treat the effluent with reaction condition. In the optimization of decolorization process four factors viz, pH, Laccase LCC2, Temperature and HBT in three levels with an OA layout of L9 (3^4) were selected. The obtained decolorization percentage from nine experiments designed by the methodology was analyzed to determine the optimum parameters using "Bigger is better" as quality character. The contribution of selected factors for the successful decolorization of effluent was as follows HBT 69%, temperature 17.5%, pH 7.47% and laccase LCC2 6%. The untreated and treated textile effluents were analyzed by FT-IR and UV–Vis techniques and their toxicity with respect to Allium cepa root inhibition was measured to demonstrate the potential of laccase in the detoxification and bioremediation process. The optimized reaction condition not only successfully decolorizes the textile effluent and also detoxifies the hazardous compounds present in the effluent in an eco-friendly way.

KEYWORDS: *Pleurotus ostreatus* IMI 395545. Laccase LCC2. Textile effluent. *Allium cepa*

INTRODUCTION

Synthetic dyes are extensively used to color their products in the food, leather, paper, printing, pharmaceuticals, cosmetics, plastics and textile industries which results in the generation of large amounts of highly polluted effluents. Approximately 10,000 different dyes and pigments are used in textile and printing industries. About 8×10^5 tons of these dyes are produced annually worldwide and at least 10% of the used dyes are discharged into the environment and most of them are recalcitrant[1, 2, 3].

Dye-containing effluents are hardly decolorized by conventional biological wastewater treatments. In addition to their visual effect and their adverse impact in terms of chemical oxygen demand (COD), some synthetic dyes cause allergy, dermatitis and skin irritation and they are toxic, mutagenic and carcinogenic in humans [4, 5, 6]. Another problem is also encountered in the application of synthetic dyes which have a complex aromatic molecular structure and are designed to be resistant to physical, chemical and microbial fading [4]. Because the recalcitrant nature of the dye compounds and the difficulties of treating dying wastewater by conventional treatment methods, the removal of color from textile wastewater is one of the major environmental problem.

Enzymatic methods that are used to solve these problems generally have low energy requirements, are easy to control, can operate at high and low contaminant concentrations. Further they operate at wide pH, temperature and salinity range and have a minimal environmental impact [7, 8, 9]. Nowadays, laccase-based treatments have received much attention for the degradation of different recalcitrant pollutants and dyes[10]. 1-Hydroxybenzotriazole (HBT) is one of the most efficient laccase mediators but its high cost and potential toxicity are important drawbacks. Therefore, the optimization of HBT concentration is important for effective decolouration[11]. Furthermore the decolorization of dyes would be more attractive using this cheaper enzyme under optimum process conditions. Recently, several research works have been reported on the decolorization of various textile dyes and effluent with lignocellulolytic enzymes [12,13].

The aim of the present study was to optimize the decolorization of the recalcitrant textile effluent by purified laccase (LCC2), obtained from the *Pleurotus ostreatus* IMI 395545, using Taguchi DOE methodology.

MATERIALS AND METHODS

Chemicals

HBT, ferrous ammonium sulfate, potassium dichromate and ferric chloride were purchased from s d fine-chem Limited, India. Unless otherwise stated all chemicals were of analytical grade.

Effluent collection

Surface water samples were collected from the point of effluent drainage pipe outlet near Cauvery river new bridge in Pallipalayam, Erode, Tamil Nadu, India. Water samples were collected with optimum care in pre-cleaned polythene containers and stored at room temperature. The collected effluent was filtered through Whatman No. 1 filter paper and then sterilized by membrane filter (45µm). The color of the textile effluent was bluish green and shows strong unpleasant odour.

Laccase production

Laccase production was carried out in the optimized production medium[14] in the bioreactor ADI 1025 Bioconsole under optimized culture condition.

Purification of laccase isoenzyme and determination of molecular mass

In order to determine the number of laccase isoenzymes produced by *Pleurotus ostreatus* IMI 395545, the crude culture was centrifuged at 6000 x g for 30 min and the obtained supernatant was used for further studies. Native-PAGE was carried out according to the method described by Gabriel[15] using 5 mM guaiacol in 100 mM sodium acetate buffer [pH 6.0] at room temperature. The method for the laccase purification was adopted from a protocol described by Das *et al.* [16] with minor modifications. All operations were performed at 4 °C unless otherwise mentioned.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [17]. The same was used to monitor the development of the purification process, to determine the homogeneity and apparent molecular mass of the purified laccase. SDS-PAGE was carried out on a 4% w/v stacking gel and 10% w/v separating gel. The approximate molecular mass of the laccase was determined by calibration against broad range molecular weight markers, which contained the proteins β-galactosidase (116.25 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa). SDS–PAGE and native PAGE revealed the presence of two proteins. Non-denaturing PAGE was performed to ascertain which protein correlated to laccase activity. The non-denaturing gel was bisected and half was stained with Coomassie Brilliant Blue R-250, the other half was stained with guaiacol to determine which band correlated to laccase activity.

Laccase assay

Laccase activity was determined using guaiacol as the substrate according to the method of Sandhu and Arora [18]. Enzyme activity was expressed in units (U = µM/min/l), defined in terms of the number of µM of guaiacol converted per minute per liter (ε = 21,600 µM/min/l).

Taguchi DOE methodology

Among various statistical experimental designs, Taguchi experimental design offers distinct advantages by which many factors can be examined as well as much quantitative information can be extracted concurrently with a few experimental trials [19]. Taguchi DOE is a factorial-based approach, which has gained beyond importance recently for its application in optimizing biochemical processes. The approach involves the study of a given system by a set of independent variables (factors), both controllable and uncontrollable (dynamic/noise), over a specific region of interest (levels) [20].

Experimental design

The first step in the methodology was to determine various important factors to be optimized in the decolorization process. Based on the obtained experimental data from our initial studies and earlier reports of various scientists [21, 11, 22] four factors were selected for the decolorization of textile effluent using purified laccase (LCC2) secreted by *Pleurotus ostreatus* IMI 395545 (Table 1). The second step was to design the matrix experiment and define the data analysis procedure with an inner and outer array. In total, nine experiments were conducted with different combinations of four factors to determine the decolorization percentage.

Dye decoloration was calculated by means of the formula:

$$D = \frac{(A_{ini} - A_{obs})}{A_{ini}} \times 100$$

where *D* is the decoloration of textile effluent (in %), A_{ini} is the area under the curve of the absorption spectrum from 400 to 800nm at time zero and A_{obs} is the area under the curve of the absorption spectrum from 400 to 800nm at a determined time.

Qualitek 4-Software

In the present study an automatic design of experiments using Taguchi based Qualitek-4 software (Nutek Inc., MI) was used. The obtained experimental data was processed in the Qualitek-4 software with bigger is better as quality characteristics for the determination of the optimum reaction condition for the maximum decolorization of textile effluent.

UV–Vis analysis

The dye degradation products produced during biodegradation of effluent by laccase alone and laccase in presence of redox mediator were studied by following the changes observed in the UV–Vis spectra (from 350 to 700 nm) using a UV–Vis spectrophotometer (Systronics 118).

FT-Infrared spectrum analysis.

The absorbance FT-IR spectra of the untreated and treated textile effluent were recorded using an FT-IR Spectrum (2000 Perkin–Elmer) spectrometer. The spectra were collected within a scanning range of 400–4000 cm^{-1}. The FT-IR spectrum of the control was finally subtracted from the spectra of the untreated and treated effluent.

Effluent chemical analysis

Characteristics of the pooled textile effluent were analyzed for three data sets and values were calculated for representative data. Analysis included pH, color, total suspended solids (2540 C), total dissolved solids (2540 D), BOD (5210 B) and COD (5220 B) were determined using standard methods of American Public Health Association, American Water Works Association, Water Environment Federation [23].

Allium cepa linn assay

The Allium test provides a rapid screening procedure for toxic chemicals and pollutants which may represent environmental hazards. *Allium cepa* root inhibition test and microscopic analysis of root tips were carried out by the method of Akintonwa *et al.*[24]. Root growth inhibition and undesirable effects on chromosomes provides an initiation of possible toxicity. Healthy equal sized onions were obtained from local market of Erode, Tamil Nadu, India. The dried outer scales were carefully removed leaving the ring of the root primordial intact[25]. Five onion bulbs were utilized for each concentration tested; filtered effluent was diluted with good quality of tap water to obtain two different dilutions (1:1 and 1:4 v/v). The tap water of good quality was used as control. Undiluted textile effluent was also used for experiments. The base of each of the onion bulbs was grown on the effluent inside a 35 ml boiling tube and placed away from sunlight for 4 days after which the root length was measured. The test solutions were used at room temperature and at the termination of the exposure, the lengths of the root bundles were measured and their mean ± SD were calculated.

Microscopic analysis

The root tips at a length of 10 mm were cut off and fixed in acid: alcohol solution (1:3) by heating for 5 min at 50 °C. Thereafter the terminal root tips (1-2 mm) were cut off and squashed on the slide and stained with eosin solution for 10 min. The cover slip was then carefully lowered on the stained area to avoid air bubble and slides were carefully dampened with the use of a filter paper to remove the excess stain. The cover slip was fixed carefully to the slide with nail varnish. The slides were examined under the microscope to determine the mitotic index. The Mitotic Index (MI) was determined by counting all stages of mitotic cells out of 1000 cells:

$$\text{Mitotic index} = \frac{\text{No. of dividing cells}}{\text{Total number of cells analyzed}} \times 1000$$

RESULTS AND DISCUSSIONS

The growth of the fungus and the production of enzyme were highly influenced by effective controlling the factors like pH, temperature and percentage of dissolved oxygen tension (DOT) provision available in the bioreactor. At the end of 10th day, the production of laccase *Pleurotus ostreatus* IMI 395545 reached the maximum activity (data not shown) in the optimized medium[14]. The native PAGE results revealed that two laccase isoenzymes (LCC1 and LCC2) were extracellularly produced by *Pleurotus ostreatus* IMI 395545 (Fig 1). Mansur *et al.*[26] reported that two laccase (LCC1 and LCC2) were purified by simple purification steps like ammonium sulfate precipitation DEAE and Biogel chromatography.

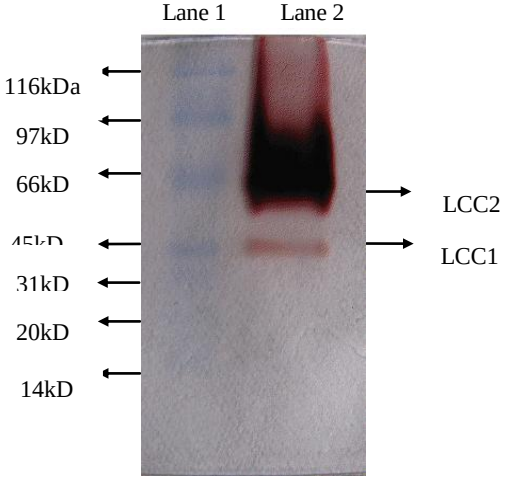


Fig 1: Zymogram analysis of laccase activity of *Pleurotus ostreatus* IMI 395545 in native PAGE. Lane 1- Protein marker; Lane 2- Zymogram analysis of laccase isoenzymes (LCC1 and LCC2) on native-PAGE by guaiacol.

Pleurotus ostreatus IMI 395545 laccase LCC2 isoenzyme was purified to homogeneity from the culture filtrate using four step purification procedures as summarized in Table 1. LCC2 was used in our present as this is the dominant and easily purified enzyme when compared to LCC1. In anion exchange chromatography DEAE-Cellulose column, two enzyme peaks were eluted obtained by linear gradient elution. LCC1 and LCC2 were eluted at approximately 660 nm and 680 nm concentration of NaCl. In ammonium sulfate precipitation, the specific activity was increased to 3.50 U/mg protein and the yield was 65.8% with a purification factor of 1.44 fold. The dialyzed sample was applied to a DEAE-cellulose column. In DEAE cellulose column chromatography, the specific activity was increased to 7.16 U/mg proteins and the yield was 50.5% with purification factor of 2.95 fold. The yield of purified laccase by this method was higher when compared to other reports in Pleurotus species [26, 16].

Table 1: Summary and procedure of *Pleurotus ostreatus* IMI 395545 extracellular laccases

Purification steps	Volume (ml)	Total laccase activity (U)	Total protein (mg)	Specific activity of laccase (U/mg)	Purification Fold	Yield (%)
Crude enzyme (culture filtrate)	1000	850	350	2.42	1.0	100
Ultrafiltration	100	780	240	3.25	1.34	91.7
Ammonium sulfate precipitation (80%)	25	560	160	3.5	1.44	65.8
DEAE-Cellulose LCC1 isoenzyme* LCC2 isoenzyme	 4 15	 35 430	 NE 60	 NE 7.16	 NE 2.95	 4.1 50.5
Biogel P-200 LCC2 isoenzyme	10	335	30	11.16	4.8	39.41

NE- Not estimated

Generally textile effluents containing recalcitrant dyes are not completely degraded or decolorized in the presence of enzymes like laccase. Addition of certain redox mediator enhanced the range of substrates and efficiency of degradation of compounds[27]. The first step in the optimization of effluent decolorization was the identification of suitable factors that affected decolorization and their range. Decolorization experiments were performed in the effluent by employing selected 18 experimental trails in combination with 4 factors at three levels (Table 2) and the results obtained from each set was defined as the decolorization percentage and was shown in Table 3. Decolorizaion of the effluent were found to be very much dependent on the reaction condition. The average effect of the factors along with interactions at the assigned levels on the effluent decolorization by purified laccase LCC2 was shown in Table 4. Individual factors at the level stage, laccase LCC2 has highest effect in level 1 whereas HBT has higher effect in level 2 and 3. The difference between level 2 and 1 of each factor indicates the relative influence of the effect. The larger the difference, the stronger is the influence. According to the Table 4, that among the factors studied, HBT showed stronger influence compared to other three factors. The above statement was in good agreement with the report of Murugesan *et al*.[28] where HBT was essential for dye decolorization and optimization of HBT was independent of dye concentration. The decolorization percentage of the effluent was increased when the concentration of the HBT was increased up to level 2 but further increase leads to decrease in the effluent decolorization. Soares *et al*. [29] reported that excessive amount of HBT may produce radicals which affect the activity of the enzyme. Moreover, the redox mediators are effective in some circumstances; at high concentrations they tend to inhibit the laccase activity[30]. The above data and arguments suggests that optimum amount of HBT is needed for successful reduction of residual dye in the effluent.

Table 2: Selected factors and assigned levels for effluent decolorization

S.No	Factors	Level 1	Level 2	Level 3
1	pH	4.0	5.0	6.0
2	Laccase LCC2 (U)	1.5	3.0	4.5
3	Temperature (ºC)	30	60	70
4	HBT (mM)	0.5	1.0	1.5

Table 3: L9 (3^4) orthogonal array of designed experiments

Experiment No	Column				Decolorization percentage*
	1	2	3	4	
1	1	1	1	1	21.20±0.2
2	1	2	2	2	75.30±0.5
3	1	3	3	3	76.50±0.4
4	2	1	2	3	76.60±0.6
5	2	2	3	1	55.25±0.8
6	2	3	1	2	75.60±0.5
7	3	1	3	2	77.43±0.4
8	3	2	1	3	64.39±0.3
9	3	3	2	1	54.39±0.7

Table 4: Effects of selected factors at different levels

Serial No	Factors	Level 1	Level 2	Level 3	L2-L1
1	pH	57.666	69.155	65.422	11.489
2	Laccase LCC2 (U)	58.411	65	68.833	6.588
3	Temperature (ºC)	53.744	68.766	69.733	15.022
4	HBT (mM)	43.622	76.111	72.511	32.489

Increasing the units of laccase LCC2 and temperature has resulted in increase in effluent decolorization. But the difference between the level 2 and 3 was negligible. Understanding the interaction between two factors gives a better insight into the overall process investigation. Any individual factor may interact with any other factors creating the possibility of a large number of interactions. In the Table 5, estimated interaction severity index (SI) of the factors under study helps to know the influence of two individual factors at a range of levels of the interactions. The highest SI (67.07) was observed when pH interacts with Laccase LCC2, followed by pH and temperature (47.21). Interaction of pH [5.0] with the above factors plays important role in the maximum effluent decolorization. Young and Yu[31] agreed that most of the dyes need acidic conditions (pH 3.5–5).

Table 5: Interaction of severity index for different factors

Serial No	Factors	Columns	SI (%)	Reserved columns	Levels
1	pH × Laccase LCC2	1 × 2	67.07	3	[3,1]
2	pH × Temperature	1 × 3	47.21	2	[3,3]
3	Laccase LCC2 × Temperature	2 × 3	39.59	1	[1,3]
4	Laccase LCC2 × HBT	2 × 4	32.18	6	[1,2]
5	pH × HBT	1 × 4	30.02	5	[3,2]
6	Temperature × HBT	3 × 4	29.78	7	[3,2]

Fig 2 shows the untreated and treated textile effluent. The obtained experiment results were in good agreement with the report of khlifi *et al*. [32], where redox mediator such as HBT was required to decolorize the textile industry effluent. Further, Hadibarata and Tachibana[33] proved that addition of HBT to purified laccase increases the rate of decolorization. The extent of the textile effluent decolorization was analyzed by UV-Vis and FT-IR spectrum analysis. Fig 3 shows the disappearance of the typical absorption peak at 600 nm. It confirms the significant reduction of residual dye in the effluent. Fig 4 shows the FT-IR spectra obtained from the untreated effluent sample showed several peaks in the region where couplings of C-O and C-C stretching are normally observed (1000-1500 cm^{-1}). When the effluent was treated under optimized condition, there was a significant reduction in intensity of the absorption spectra in the same region.

A B C

Fig 2: Control textile effluent (A) textile effluent treated with laccase (LCC2) (B) and textile effluent treated with optimized laccase (LCC2) and HBT concentrations (C).

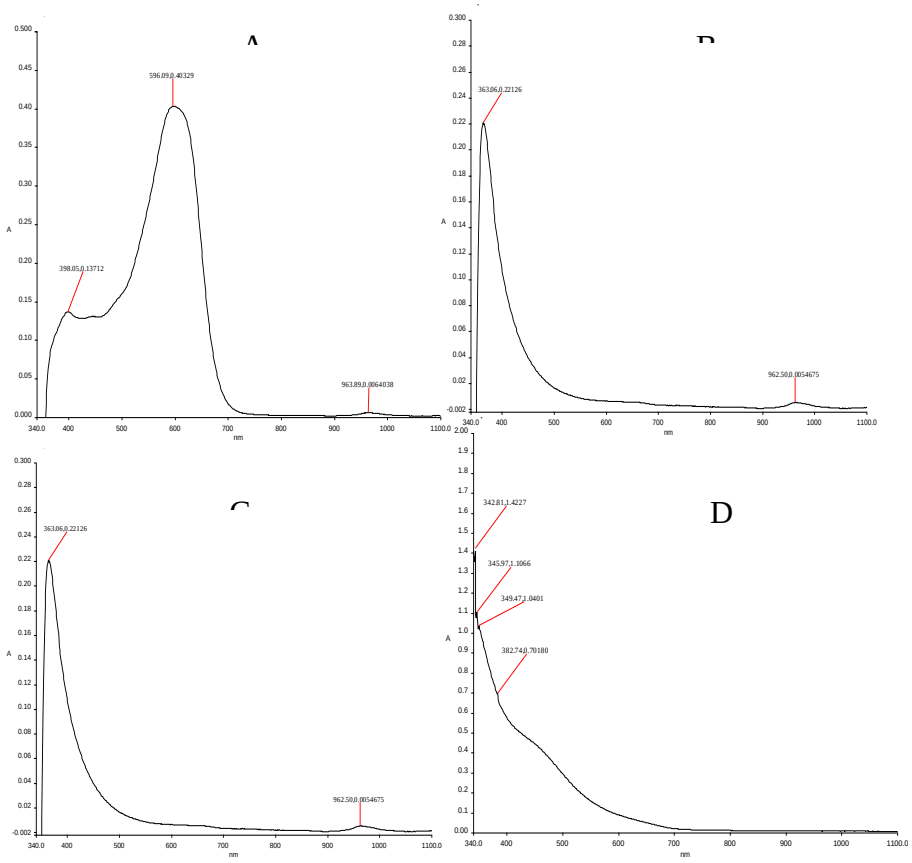


Fig 3: UV-Visible absorption spectra of the reduction of residual dye in the textile effluent by *Pleurotus ostreatus* IMI 395545 purified laccase. (A) Diluted effluent (1:4 dilution), (B) laccase (LCC2) (0.5 U), (C) HBT (0.5 mM) and (D) Treated effluent (1:4 diluted effluent + 0.5 U Laccase (LCC2) + 0.5 mM HBT).

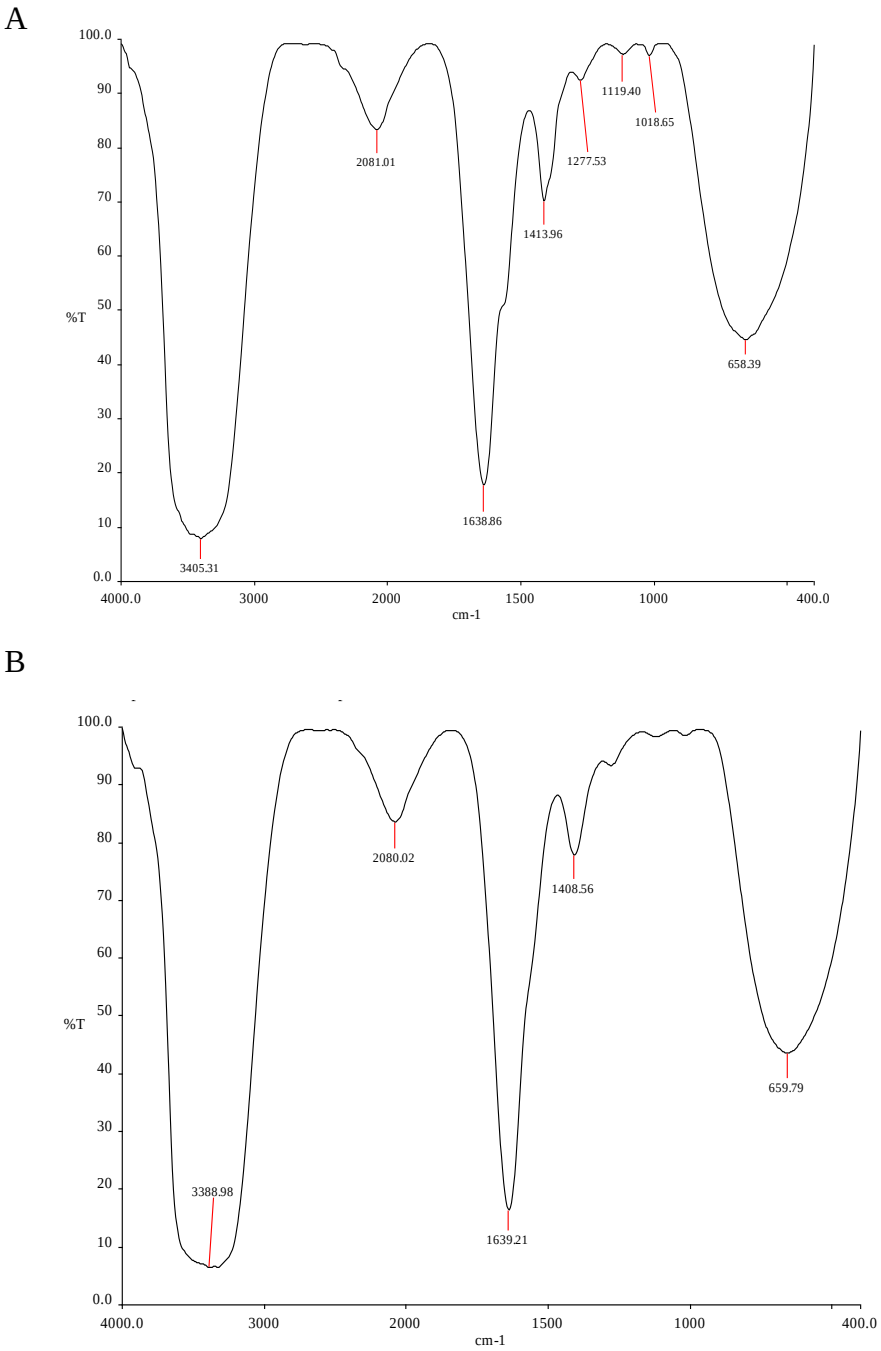


Fig 4: FT-IR absorption spectrum of untreated textile effluent (A) and textile effluent treated with optimized laccase (LCC2) and HBT concentrations (B).

According to the table 6 most influential factors in the decolorization was the HBT, accounting for 68.99% followed by temperature (17.48%), pH (6.0%), and laccase LCC2 (7.5%). It is evident that, upon considering the optimum reaction condition from the experiments designed the decolorization percentage of the effluent can be increased from 64% to 91.5% (predicted value). Under experimental condition decolorization of 85% was achieved (Table 7). The results indicate a good adequacy of the predicted model. The obtained percentage of effluent decolorization was higher than the one reported by Damle and Shukla [34].

Table 6: Analysis of variance (ANOVA).

Serial no	Factors	DOF (f)	Sum of squares	Variance	F-Ratio	Pure Sum	Percent
1	pH	2	618.242	309.121	17707.64	618.207	7.471
2	Laccase LCC2	2	500.2	250.1	14326.69	500.165	6.044
3	Temperature	2	1446.733	723.366	41437.171	1446.698	17.483
4	HBT	2	5709.171	2854.585	163521.454	5709.136	68.995
5	Other/ Error	18	.314	.017			.007
6	Total	26	8274.662				100.00%

Table 7: Optimum of decolorization conditions and their contribution

S No	Factors	Values	Levels	Contribution
1	pH	5.0	2	5.074
2	Laccase LCC2(U)	4.5	3	4.751
3	Temperature(°C)	70	3	5.651
4	HBT (mM)	1.0	2	12.029

The obtained results in the Table 8 proved that optimized laccase treatment not only reduce the residual dye in the textile effluent but also decrease the BOD and COD level. The above statement was in good agreement with the report of Arulmani *et al.* [35] where the laccase plays an important role in the reduction of BOD and COD levels in the textile effluent treated with immobilized mycelium of *Pleurotus sajor-caju*. The removal efficiency of chemical oxygen demand (COD) from pulp and paper wastewater using laccase-polymerized membrane filtration process was reported by Ko and Fan [36].

Table 8: Physico-chemical properties of untreated and treated textile effluent with optimized laccase and HBT concentrations

S No	Parameters	Untreated effluent	Treated effluent
1	pH	8.52	6.45
2	Color	Dark Blue	Light brown
3	TSS (mg/dl)	9	-
4	TDS (mg/dl)	612	275
5	BOD (mg/dl)	452	162
6	COD (mg/dl)	1370	640

The toxic effect of textile effluent has direct correlation with the root length inhibition and decreased mitotic index of *Allium cepa* (Table 9). The above obtained results have good concurrence with the *Allium cepa* root inhibition by pharmaceutical effluent reported by Akintonwa *et al* [24] and Samuel *et al.* [37]. Osma *et al.* [38] demonstrated the toxicity of the textile effluent in ryegrass seeds root inhibition test. Although several species have been traditionally used for evaluating phytotoxicity, there are no standardized seed species in use worldwide[39]. As the dilution decreased from 1:4 dilution to undiluted effluent, the mean root length and mitotic index decreased considerably. In the effluent treated with laccase under optimized reaction parameters both the root growth and mitotic index increased when compared to the untreated textile effluent (Fig 5 and 6). The observed chromosomal structural abnormalities may be due to toxicity of the textile effluent to the root of *Allium cepa*. The resulted altered structure of the chromosomes may due to the interference of the effluents with the process of mitotic division and with the mitotic spindles. The above results are in good agreement with the earlier report of Akinboro and Bakare [40].

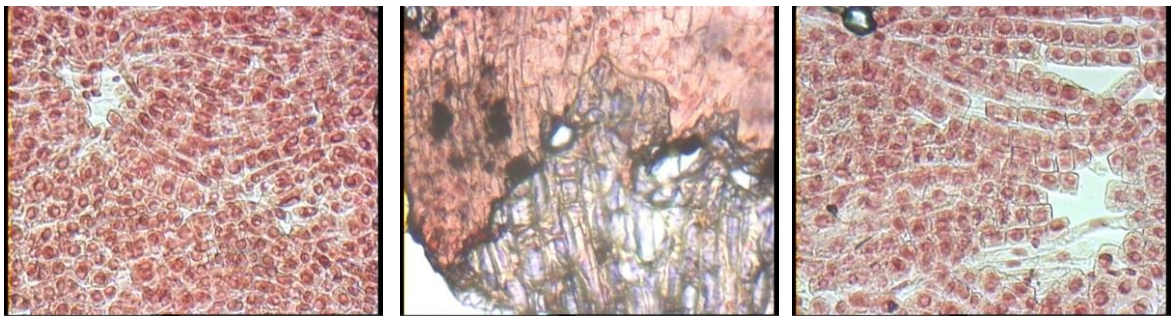
Table 9: Effects of untreated and laccase treated textile effluent on the cytology and root growth of *Allium cepa*

Serial no	Particulars	No of the dividing cells	MI	Root length ± SD (cm)
1	Control	52	115	4.36±0.26*
2	Undiluted effluent	24	55	0.85±0.32*
3	1: 1 dilution	28	58	2.05±0.30*
4	1: 4 dilution	32	67	3.54±0.28*
5	Laccase treated undiluted effluent	46	110	4.08±0.31*

Dilution with distilled water, *p<0.05 (compare with control)

A B C

Fig 5: Root length of of *Allium cepa* after 3 days incubation in: (A) water (control), (B) untreated textile effluent and (C) textile effluent treated with optimized laccase (LCC2) and HBT concentrations.



A B C

Fig 6: Onion root tip stained with acetyl carbinayl stain cultured in normal tap water (A), untreated effluent (B) and textile effluent treated with optimized laccase (LCC2) and HBT concentrations (C).

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CONCLUSION

The Taguchi method has provided a systematic and efficient approach to understand complex effluent decolorization process for the optimization of the near optimum design parameters, only with a few well-defined experimental sets. The optimization can be easily validated with the impact of factors contributed for the above process. The optimization of temperature, pH and mediator concentration show the great potential of purified laccase in mediator treatment under the conditions used. These results suggest that LMS could be used for treating textile industry effluent. According to the results of this study and other reports, it is clear that decolorization ability of the purified laccase can be substantially increased by carefully optimizing the operational conditions such as pH, concentration of redox mediator HBT and temperature. This study showed the use of Taguchi DOE methodology and statistical optimization tools, which enables us to find the optimum levels of the most significant variables for color removal with minimum effort and time. The above experiment finds a promising way to treat the polluted water in an eco-friendly way without dumping hazardous materials in soil.

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