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DECOLOURIZATION OF INDUSTRIAL DYES USING A WHITE ROT FUNGI *LENTINUS EDODES*

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ABSTRACT
Different species of white rot fungi are known to be excellent candidates for bioremediation of various organo-pollutants like polycyclic aromatic hydrocarbons, pesticides, amines , dyes etc. Synthetic dyes are widely used in various industries like textile, paper, leather, food , plastic etc. The discharge of toxic effluent from these industries into water bodies affects the aquatic life and environment. White rot fungi are known for the synthesis of extracellular ligninolytic enzymes which are involved in degradation of ligninocellulosic substrates. In this study, decolourization of two industrial dyes Rhodamine B and Remazol Brilliant Blue was carried out by using crude enzyme extract from Lentinus edodes. Quantitative estimation of dye decolourization using whole fungal mycelium was also done. Activity of the major ligninolytic enzymes like laccase, lignin peroxidase & manganese peroxidase enzymes was monitored. Extent of dye decolourization was observed by varying parameters like substrate, pH, dye concentration and volume of enzyme extract. The efficiency of dye degradation was further established by comparing the differences in FTIR spectra and HPLC analysis of the degraded metabolites & the standard dye spectra . Phytotoxicity assessment of the degraded products was monitored by measuring effect on germination of Phaseolus mungo. The study found that Lentinus was able to decolourize RBBR and Rhodamine upto 74%and 72% respectively after 8 days of incubation.

Keywords: Lentinus, ligninolytic enzymes, bioremediation, synthetic dyes

1. INTRODUCTION
One of the serious environmental issues all over the world in current times is the pollution of water occurring from factories and industries . During the dyeing process ,the textile industries consume large amounts of water and produce highly coloured effluent that contains unused dyes and other chemicals (Dhir,2022).

It becomes a major cause of water and soil pollution in developing and underdeveloped countries. The synthetic dyes released from these industries lead to major environmental disruption by contaminating the aquatic bodies and adversely affecting human health. Many of the dyes are persistent and not easily degradable. Based on the chromophores structure, the synthetic dyes are classified into several groups, like azo, indigo,anthraquinone, triphenylmethyl and phthalocyanine dyes(Isanapong and Mataraj, 2018).

Remazol Brilliant Blue R (RBBR) dye is an anthraquinone based vinylsulphone dye used in the textile industry for dyeing cellulosic fibers. It is frequently used as the starting material in the production of polymeric dyes. Rhodamine B (Basic violet 10, C.I. 45170) is an important fluorescent cationic textile dye. Due to its cationic nature it binds to anioinic fabrics which contain negative charges such as polyester fibers, wool, silk, and acrylic fibers. Its structure is comparatively rigid than other organic dyes which makes it important from bioremediation point of view.

Several physical and chemical processes like adsorption membrane filtration, ozonation, coagulation**,** flocculation, precipitation, reduction electrochemical oxidation, photolysis and photodegradation have been utilized for the management of dye based pollution. (Krishnan *et al*., 2017). But there are certain limitations of these method like high cost of operation and energy requirement, complex and tedious procedures, incomplete degradation, and secondary waste generation. Alternately, bioremediation by is considered a clean, effective, and safe technology for detoxification of dyes from wastewater. Biological methods include use of microbes such as bacteria, fungi, yeast, algae and plants (bioremediation) (Shilpa and Shikha, 2015; Rajwade *et al*., 2022). These have drawn quite a lot of attention due to their eco-friendliness. The mechanism behind biodegradation of dyes includes adsorption on microbial biomass, biosorption and/or enzymatic degradation (Asgher *et al*., 2016). Fungi are regarded as the

best choice among the numerous biological agents due to their rapid growth, high biomass production, extensive hyphal growth, and high surface-to-cell ratio. Also several studies have proved that fungi possess the ability to degrade complex organic substances using extracellular ligninolytic enzymes like laccase, manganese peroxidase, and lignin peroxidase. Thus due to its environmental friendly and cost effective nature, biological decolourization is a promising approach (Afreen *et al*., 2018; Sing *et al*., 2017). *Lentinus edodus* is an important white rot fungus that releases ligninolytic enzymes that can break down lignin into CO_2 and H_2O.This feature makes it an excellent candidate for bioremediation of dyes that have lignin like aromatic ring structures (Paterson, 2007).In the present study the decolourization of two industrial dyes Rhodamine B and Remazol Brilliant Blue was analysed by using crude enzyme extract from *Lentinus edodus.*

2. MATERIALS AND METHODS

2.1. Procurement of mushroom culture & dyes-

The culture of *Lentinus edodes* was procured from Indira Gandhi Krishi Vishwavidyalaya (IGKV) Raipur, C.G. ,subcultured and maintained at 4 ° C. Rhodamine was procured from Drolia industries Raipur, C.G. while Remazol Brilliant Blue R was from Sisco Research Laboratories, Raipur C.G.

2.2. Screening and Quantitative estimation of dye decolourisation–

Potato dextrose agar (PDA) plates containing 20,40,60,80 and 100mg/L concentration of Remazol Brilliant Blue and Rhodamine dye were prepared for the screening of dye decolourization. All plates were directly inoculated with fungal culture and kept in incubator at 27°C (Skariyachan *et al*., 2016).For quantitative estimation of dye decolourisation, each of the dyes was added at a final concentration of 50mg/L to a three day old broth culture of *Lentinus.* Spectrophotometric reading was taken on zero day, 2nd day, 4th day, 6th day and 8th day at 557nm for Rhodamine and at 595nm of RBBR dye respectively. Percent decolourization of both the dyes was calculated by the formula given below-

$$\text{Decolourization\%} = \frac{A^0 - A}{A^0} \times 100$$

Where A^0 is the initial absorbance of the dye and A the absorbance of the dye with time. The decolourization % reflects decrease in dye concentration due to fungal mediated remediation (Ramachandra *et al*., 2013).

2.3. Ligninolytic enzyme activity determination-

Three mycelial bits (7 mm each) were inoculated in 100 mL PDB enriched with 0.1 g rice straw powder. The cultures were incubated at 27°Celsius and monitored every three days until day 15 for the development of ligninolytic enzymes, Laccase, manganese peroxidase, and lignin peroxidise (Isanapong and Mataraj ., 2018).

2.3.1. Laccase activity

Laccase activity was determined by monitoring the oxidation of ABTS at 420nm. Test solution was prepared by using 0.25ml of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 0.75 ml citrate buffer and 1.5ml crude enzyme was added to it. Oxidation of ABTS was spectrophotometrically determined by an increase in absorbance at 420 nm (Ɛ420 = 0.036 µM-1cm-1) (Sing *et al*., 2017).

2.3.2 Manganese peroxidase activity

Reaction mixture contain 0.1mg/ml phenol red, 0.1mM manganese sulfate, 1mg/ml BSA, 0.1mM hydrogen peroxide, 25mM sodium lactate and 0.5ml culture filtrate. Oxidation of phenol red at 610 nm denotes the activity of MnP.

2.3.3 Lignin peroxidase activity

Lignin peroxidase activated was estimated by the method of Tien and Kirk (1983). LiP activity was assayed by the demethylation of methylene blue at 650nm by using 32µM methylene blue in 50mM sodium tartarate buffer of pH=4, 0.1ml of 0.1mM H2O2 and 10µl of crude enzyme.

Percentage of decolourization of methylene blue was calculated as

A650 of control- A650 of test X 100

A650 of control

2.4 Optimization of different parameters-

Effect of presence or absence of substrate, dye concentration, pH and volume of enzyme extract on the extent of decolourization of Rhodamine and RBBR by the fungi was analysed. *Lentinus* was grown in PDB supplemented with and without rice husk for 7 days. For dye decolourization 2 ml of the enzyme extract was added to 20 ml of 50mg/L concentration of dye solution. Absorbance was measured on hourly basis from zero hour to six hour at 557 nm for Rhodamine and 595 nm for RBBR. Different concentrations of dye from 20,40,60,80 and 100 mg /L were prepared and 2mL of enzyme extract was added to 20 mL of the dye solution of each concentration. The effect of pH was studied by preparing 50mg/L dye concentration at 3 different pH 4, 5 and 6. Finally to analyse the effect of variation in volume of enzyme extract used, different volumes (1,2,3,4 & 5 ml)of enzyme extract was added to 20 ml of 50mg/L of each dye solution(Isanapong and Mataraj, 2018).

2.1 Screening of metabolites of dye degradation-

Fungal degradation of the two dyes was assessed by comparing the Fourier transformed infrared spectroscopy (FTIR) peak profiles of the metabolites of the dyes and those of their respective abiotic control (without fungal culture). The absorption of IR radiation at different wavelength indicates different types of bonds and therefore different functional groups. The analytical spectrum of unknown sample is then compared with catalogued of known material spectra,to identify the components. In the present work, we used 'Compact FT-IR spectrophotometer ALPHA II model of Fourier Transform Infrared spectroscopy to measure the absorbance spectra of IR radiation.The samples to be were prepared by drying them into Hot air oven for a week. Another attempt was done to identify the residual dye metabolites using the technique of High performance liquid chromatography(HPLC). LC analyses were performed on an HP 8040 M Series I LX –Nexera XR HPLC (SHIMADZU Technologies) equipped with a diode array detector. A 25-µL sample volume was introduced through a LiChrospher 100 C18 column (4 mm i.d. £250 mm length) containing 5 m packed particles (Merck KgaA, Darmstadt, Germany). The mobile phase contained 50 % acetonitrile and 50% water and was adjusted to pH 6.9. The rate was 0.5 mL min 5 at low pressure gradient and 40 °C. Chromatograms were obtained at a 254 nm wavelength.

2.5 Phytotoxicity assessment-

Phytotoxicity assessment of dyes and their metabolites was done on germination of *P. Mungo* (Bagewadi *et al*., 2017). The number of seeds germinated after treatment with untreated dye and fungi treated dye was counted after four days.

3. RESULTS AND DISCUSSION

3.1 Decolourization of dyes in solid medium

Zone clearance was observed against a dark background for both Rhodamine and Remazol Brilliant blue at 20 , 40 and 60 mg/L concentration range after a period of 6 to 7 days. In higher concentration no prominent change occurred in the period.

3.2 Decolourization of dyes in broth medium

Decolurization % increased from 32% on 2nd day to 74.6% after 8 days of incubation for RBBR. While for Rhodamine it was 21.4% on 2nd day which reached upto 72% on 8th day. In their study on decolorisation of aqueous solutions of synthetic dyes by *Lentinus polychrous* cultivated on cassava rhizoe *Boonyarit et al 2019,* found that decolorisation occurred via two mechanisms: enzymic degradation and adsorption. Their results showed that approximately 80% of Acid blue 62 was removed from the liquid phase while only 40-50% and 15% of Reactive blue 49 and Navy blue, respectively, were adsorbed on the rhizome and mycelium both after 40 to 60 hours of incubation.In a similar study Katielle *et al* (2020) used the enzyme extract from 15 day old broth culture

of *Lentinus crinitus* and got decoulrization upto 86 %, 92 % & 45 % for Reactive Blue 220 (RB220), Malachite Green (MG) & Remazol Brilliant Blue R respectively after 72 hours.

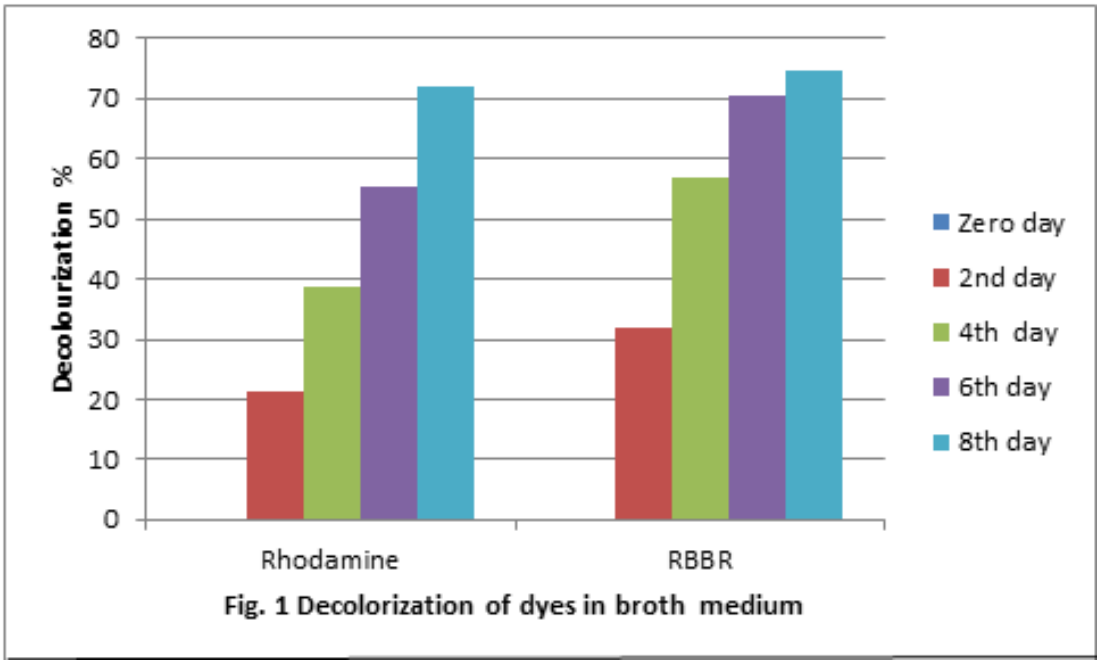


Fig. 1 Decolorization of dyes in broth medium

3.3 Enzyme activity

White rot fungi are known to be efficient producers of ligninolytic enzymes which are one of the important groups of enzymes involved in bioremediation. Spectrophotometric studies were performed for the determination of enzyme activities using standard methods. The laccase activity increased from 0.010U/mL on 3rd day to 0.090 U/mL on 15th day. While activity of Mangnese peroxidase was found to be 0.020 U/mL on the 15th day. There was not observable activity of Lignin peroxidise. Similar results were obtained by Isanapong *et al* 2018 in their study of Ligninolytic enzyme production by *L. Polychrous.* The results revealed that extracellular ligninolytic enzymes secreted by *L. polychrous* were predominantly laccase, along with low levels of manganese peroxidise ,the maximum laccase activity of 0.095 U/ml was obtained on day 15, and the activity was around six folds higher than that of manganese peroxidase. Lignin peroxidase was undetectable under the culture condition.Their study concluded that laccase for the predominant enzyme in RBBR decolourization by L. *Polychrous.*

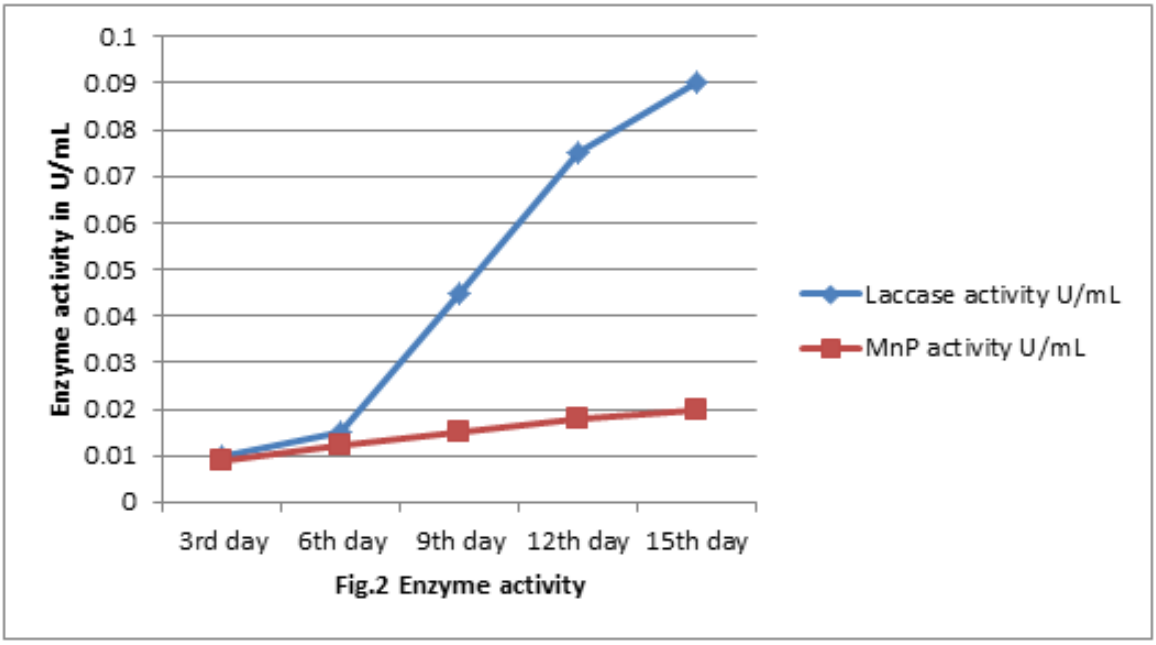


Fig.2 Enzyme activity

3.4 Effect of variation in different parameters-

Effect of presence or absence of substrate- The results showed that addition of rice husk improved the decolourization for both the dyes. The extent of decolourization percent was 48.6% & 54% for Rhodamine & RBBR respectively after 6 hours. Compared to enzyme extract taken from fungi grown without rice husk decolourized 38% & 40% of Rhodamine & RBBR respectively in the same duration.

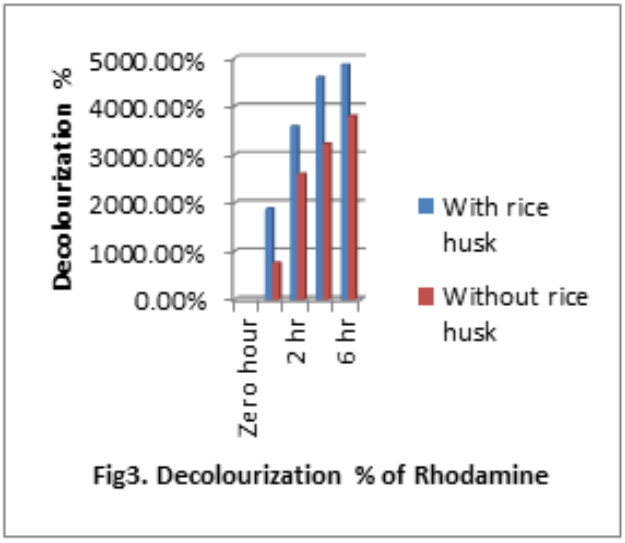


Fig3. Decolourization % of Rhodamine

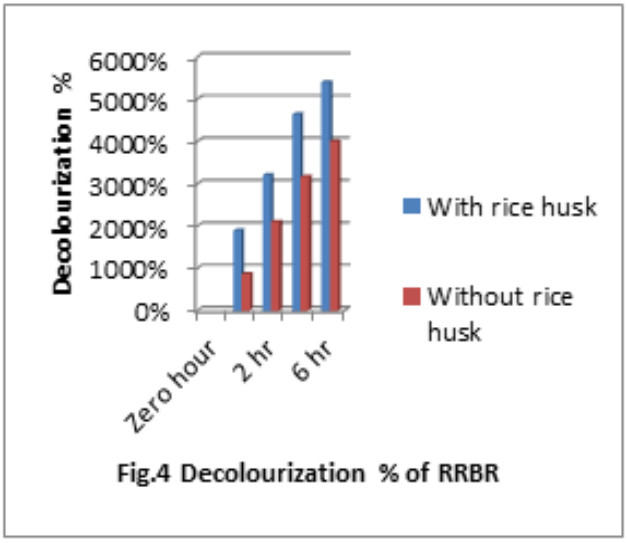


Fig.4 Decolourization % of RRBR

Effect of dye concentration-The results indicated that the percentage of decolourization decreased with increase in dye concentration as 49.6% & 52% for Rhodamine & RBBR respectively for 20 mg/L dye concentration to 18% & 19.4% for 100mg/mL of the respective dyes after 6 hours of incubation. Our results are similar to those of Hadibarata *et al*., 2011 & Isanapong et al 2018 who revealed that dye decolorization decreased with increasing concentrations of Rhodamine & RBBR, suggesting that the rate of reaction increased with the substrate concentrations until saturation.

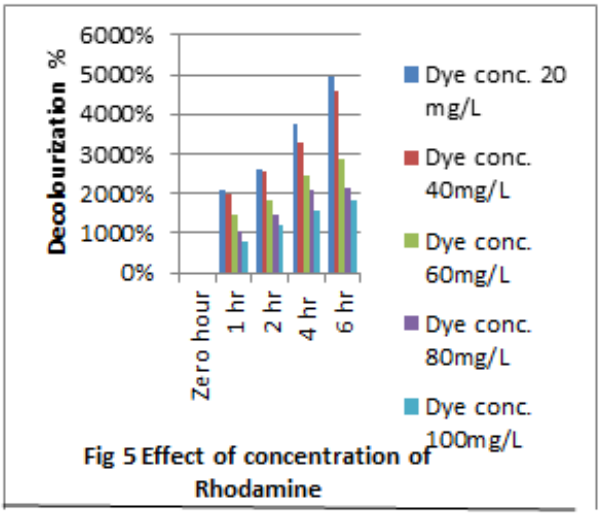


Fig 5 Effect of concentration of Rhodamine

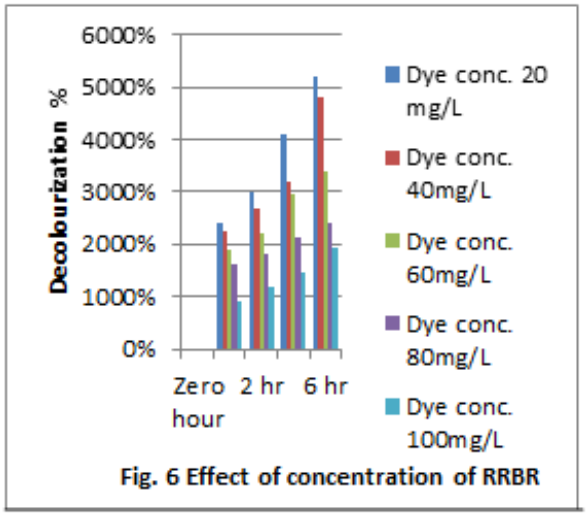


Fig. 6 Effect of concentration of RRBR

Effect of pH- The effect of pH was studies by preparing 50mg/L dye concentration at 3 different pH 4,5 & 6. After 6 hours of incubation, considerably less decolourization% was observed at pH 6 compared to almost similar Decolourization results at pH 4 & pH 5. Ratanapongleka *et al* (2014) studied the effect of pH ranging from 2 to 9 on decolorization of Acid Blue 80 by Crude Laccase from *Lentinus Polychrous.*Their study showed that The variation pH Shows a major impact on the efficiency of dye decolorization.They observed maximum decolorization efficiency around pH 5.0 beyond which the laccase decolorization activity started to decrease sharply and less than 5% decolorization was observed at pH above 8. In general, laccase enzyme tends to react differently to pH with different types of dyes.

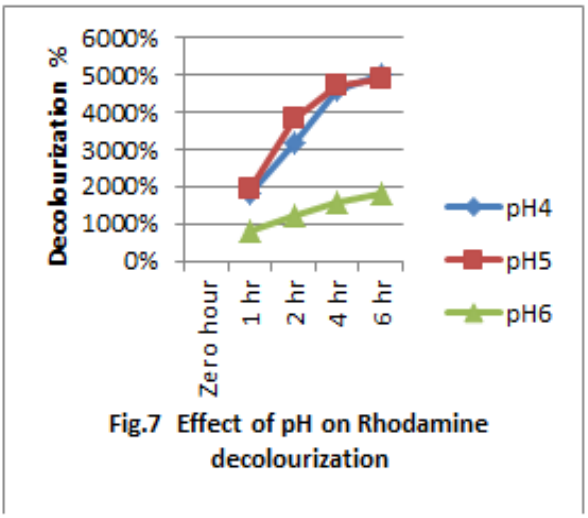


Fig.7 Effect of pH on Rhodamine decolourization

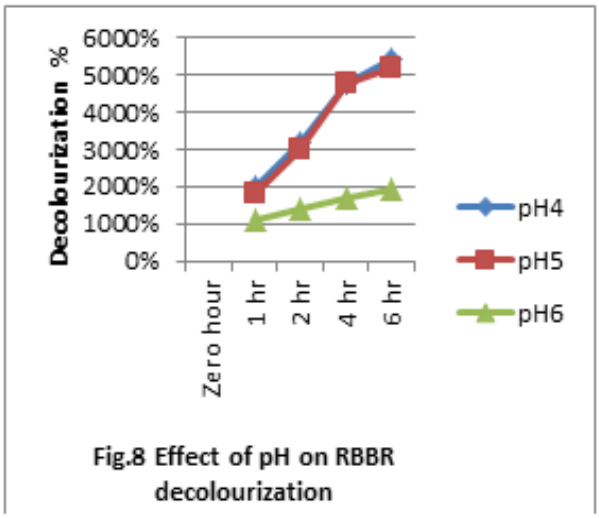


Fig.8 Effect of pH on RBBR decolourization

Effect of volume of enzyme extract -The effect of variation in volume of enzyme extract used, different volumes (1,2,3, 4 & 5 ml)of enzyme extract was added to 20 ml of 50 mg/L of each dye solution. It was observed that maximum decolourization percentage of 67% & 69.4% for Rhodamine & RBBR respectively was observed when 5mL of enzyme extract was added. Thus it can be concluded that The decolorization efficiency increased with enzyme concentrations [Ratanapongleka 2014, Isanapong *et al* 2018]. Hsu *et al*., 2012 found that 1 U/ml of laccase from *Lentinus* sp. decolorized only 29% RBBR, however, when the laccase activity was increased to 20 U/ml, the decolorization efficiency increased upto 88% [14].

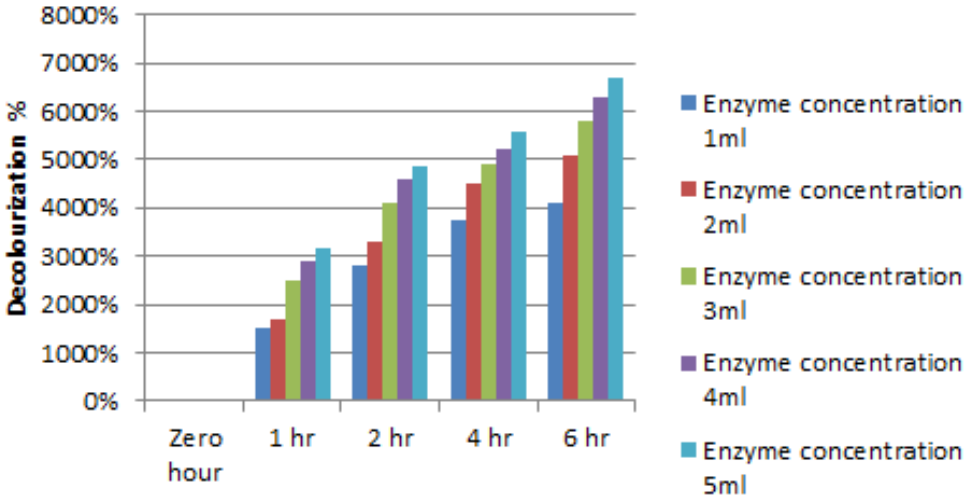


Fig.9 Effect of enzyme extract volume on Rhodamine decolourization

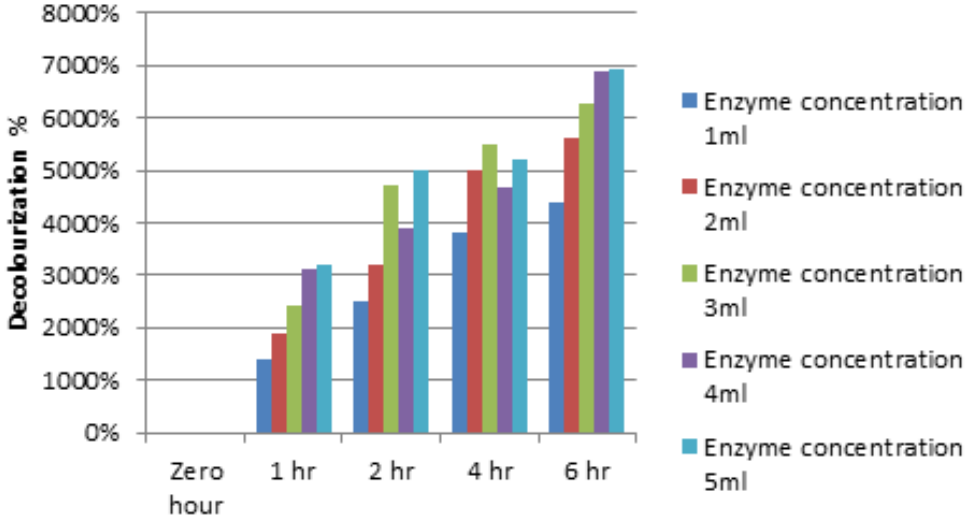


Fig.10 Effect of enzyme volume on RBBR decolourization

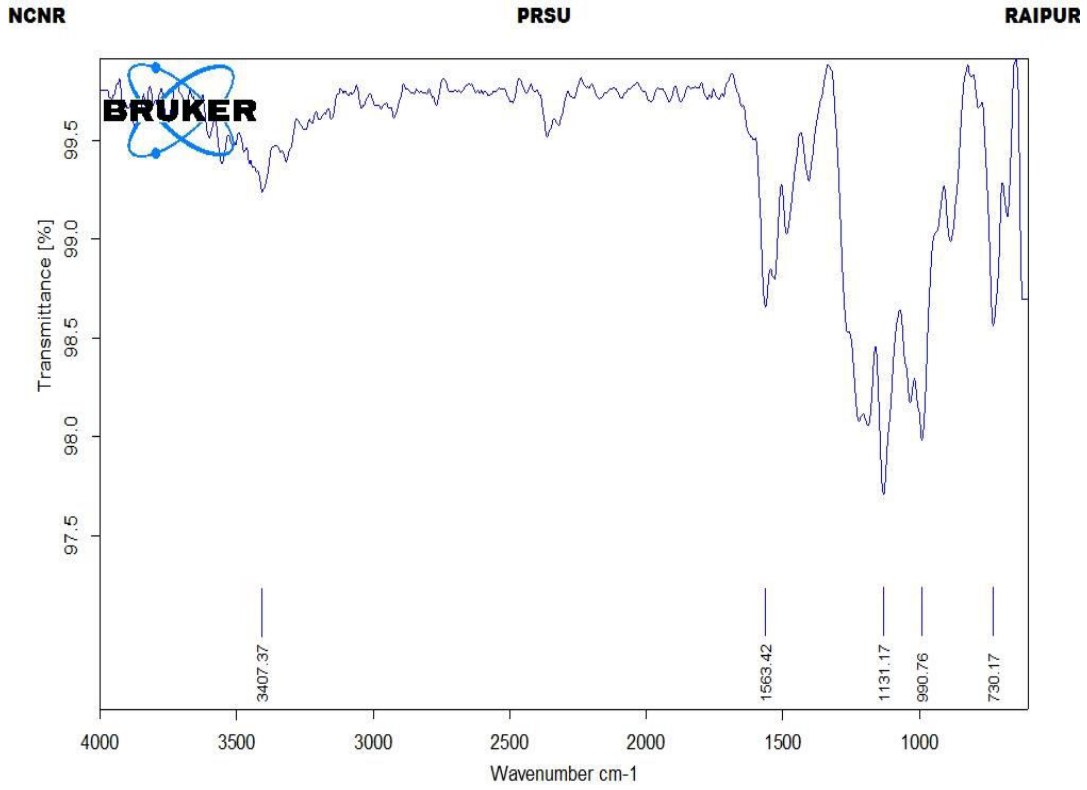


Fig. 11 FTIR spectra of RBBR before degradation with fungi (control)

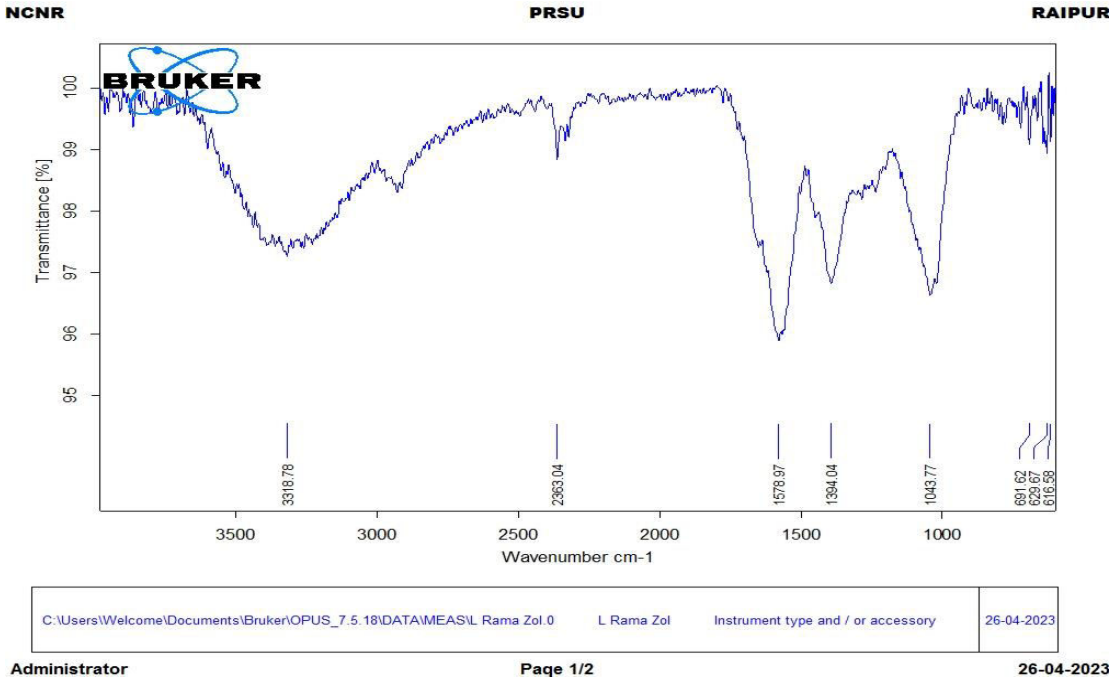


Fig. 12 FTIR spectra of RBBR after degradation with fungi

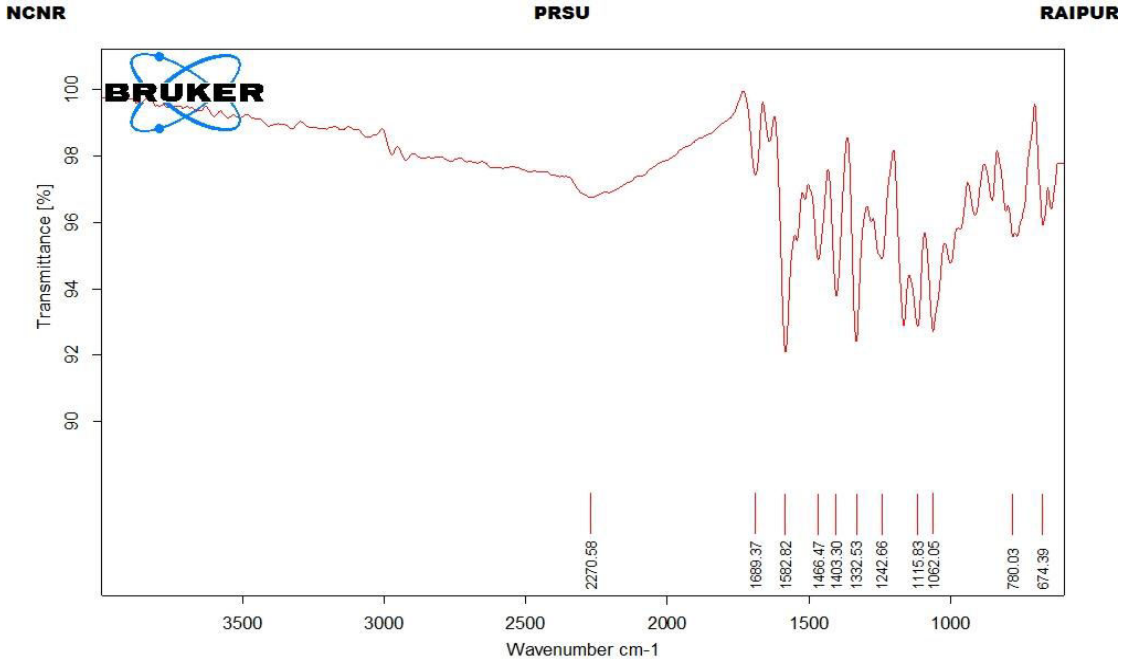


Fig. 13 FTIR spectra of Rhodamine before degradation with fungi (control)

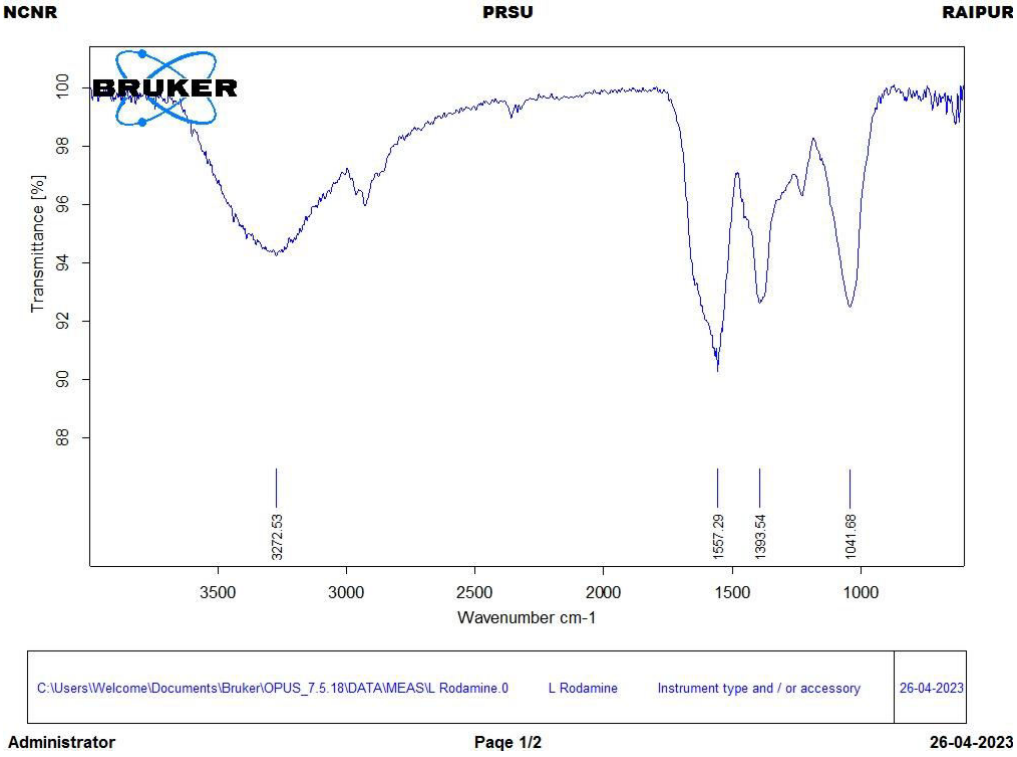


Fig. 14 FTIR spectra of Rhodamine after degradation with fungi

3.5 Analysis of Biodegraded products-

The FTIR pattern of RBBR control dye showed peaks at 3407.37 cm for N-H stretching of secondary amides, 1563.42 cm for N=N stretching of azo compounds, 1131.17 cm for C-H deformation of 1, 3-disubstituted or tri-

substituted -1 -1 benzenes, 990.7 cm for C-H ring -1 -1 deformation and 730.17 for -1 N-H deformation plus C-N stretching of amides and acyclic compounds.On the other hand FTIR spectrum of the products formed after decolorization showed different peaks at 3318.78 cm^{-1} for N-H stretching of secondary amine,2363.04 strong, broad N=C=O stretching,1578.97 N-H bending amine , 1394.04 C-H bending aldehyde,1043.77 cm strong, broad CO-O-CO stretching anhydride and multiple peaks near 691.62,629.67 & 616.58 strong C=C bending of disubstituted cis compounds.

Changes in the pattern of FTIR spectra of metabolites after fungal degradation suggest effective biotransformation of the dye RBBR. (Fig 11 & Fig 12). Similar results were seen in work of Srinivasan (2018) during bacterial degradation of RBBR. Likewise in FTIR pattern of rhodamine before degradation ,small peak occurred at 2770.58 showing C-H stretching, 1669.37 strong C=O stretching ,1582.82 medium level of C=C stretching, at 1466.47 and 1403.30 for C-H bending of methylene group, at 1332.53 ,1242.66,1062.05 for strong C-N stretching in aromatic amines, & at 780.03 and 674.39 showing strong C=C bending. After degradation a new peak was observed at 3272.53 showing O-H stretching. Further peaks were observed at1557.29 due to C=C stretching 1393.54 medium O-H bending & 1041.68 due to C-N stretching. There was a reduction in number of peaks compared to untreated dye. It shows biodegradation of dye has led to synthesis of new metabolites. (Fig 13 & Fig 14).

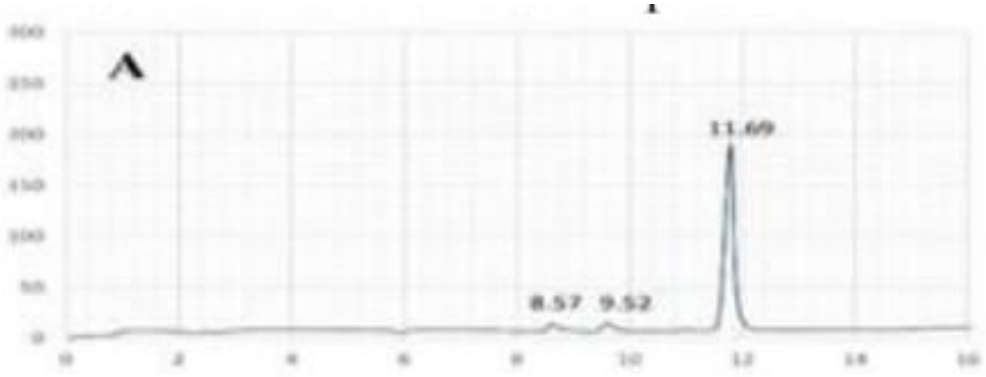
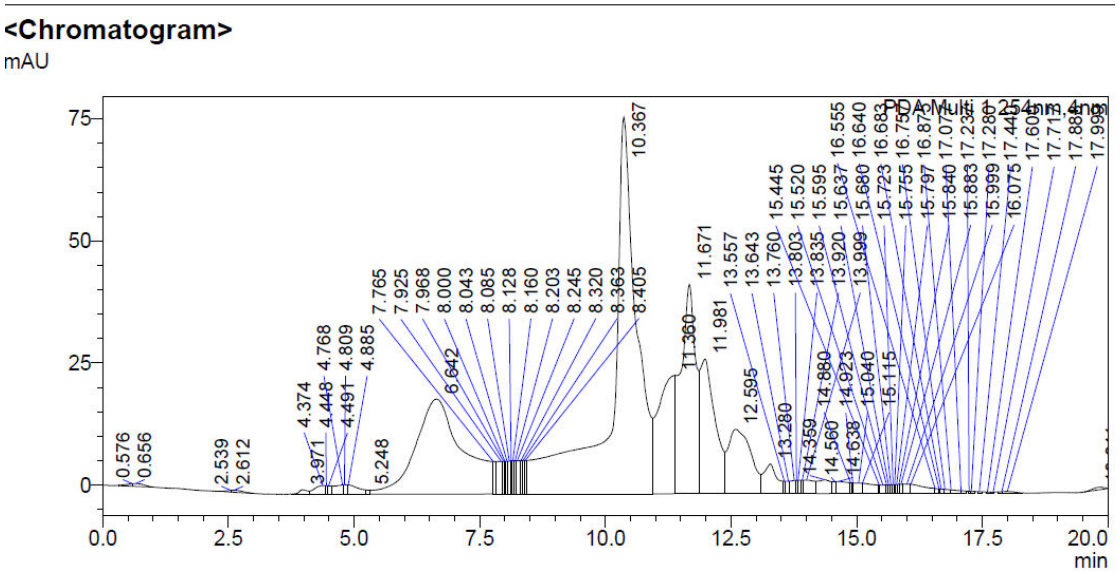


Fig .15 HPLC spectra of Rhodamine before degradation with fungi (Control)

(**Source-** Dhahir *et al* 2014)



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	0.576	5561	561	0.000		V
2	0.656	9172	634	0.000		V
3	2.539	2193	359	0.000		V
4	2.612	6765	440	0.000		V
5	3.971	12075	982	0.000		V
6	4.374	27642	1834	0.000		V
7	4.448	3401	1801	0.000		V
8	4.491	8802	1762	0.000		V
9	4.768	25369	1969	0.000		V
10	4.809	10133	2010	0.000		V
11	4.885	29309	1966	0.000		V
12	5.248	4419	914	0.000		V
13	6.642	1265842	19489	0.000		V
14	7.765	29954	6730	0.000		V
15	7.925	47052	6716	0.000		V
16	7.968	17184	6728	0.000		V
17	8.000	12925	6755	0.000		V
18	8.043	17262	6770	0.000		V
19	8.085	17328	6790	0.000		V
20	8.128	17392	6806	0.000		V
21	8.160	13091	6834	0.000		V
22	8.203	17485	6853	0.000		V

Fig 16 HPLC spectra of rhodamine after degradation with fungi

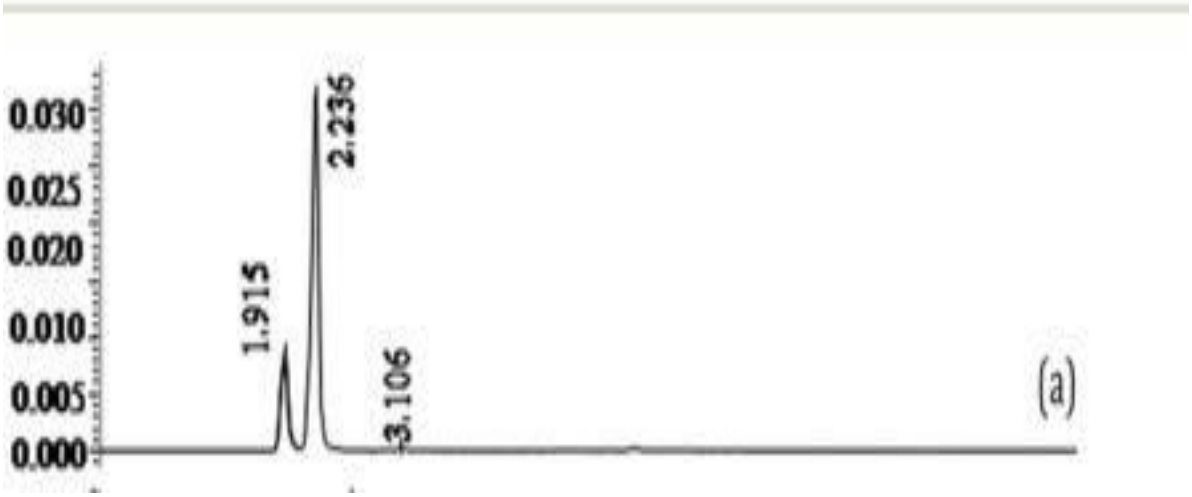
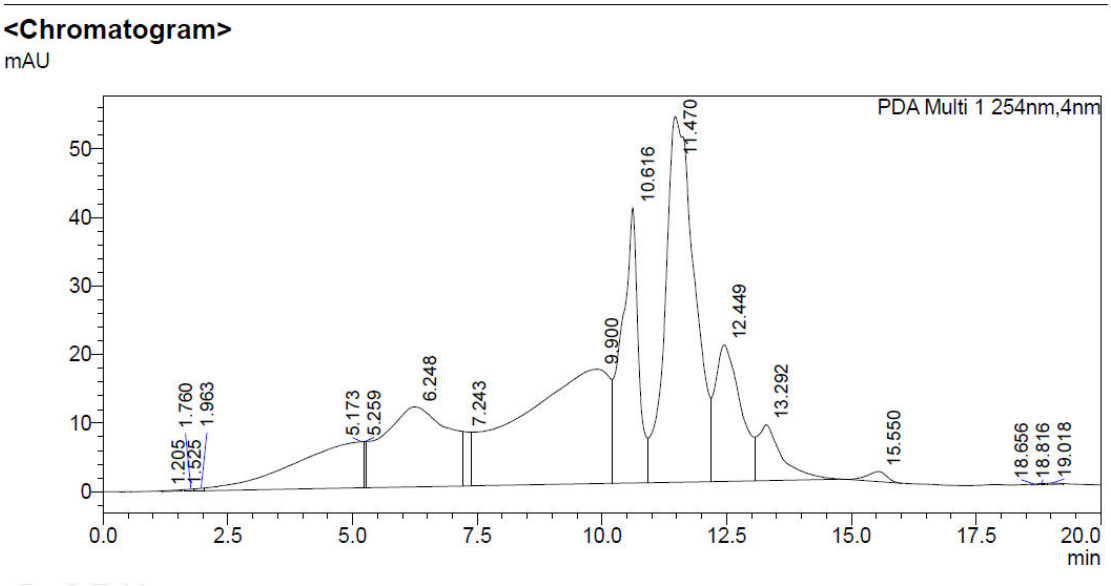


Fig. 17 HPLC spectra of RBBR before degradation with fungi(Control)

(**Source-** Kagalkar *et al* 2015)



<Peak Table>

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.205	1413	84	0.000		V
2	1.525	1125	164	0.000		V
3	1.760	2887	257	0.000		V
4	1.963	3797	357	0.000		V
5	5.173	648676	6744	0.000		V
6	5.259	17310	6767	0.000		V
7	6.248	1072191	11696	0.000		V
8	7.243	75634	7936	0.000		V
9	9.900	2052970	16695	0.000		V
10	10.616	936974	40136	0.000		V
11	11.470	2174035	53329	0.000		V
12	12.449	714592	19913	0.000		V
13	13.292	279101	8136	0.000		V
14	15.550	39601	1471	0.000		
15	18.656	1076	115	0.000		V
16	18.816	1257	160	0.000		V
17	19.018	3153	206	0.000		V
Total		8025791	174167			

Fig 16 HPLC spectra of RBBR after degradation with fungi

HPLC analysis was performed in order to confirm the degradation of Rhodamine and Remazol dye by the fungi. The profile of untreated Rhodamine shows a major peak at 11.96 min while after degradation peak shift is observed with major peaks at 3.971, 4.374 ,4.768 , 4.885 and 6.642 min with several minor peaks as well(Fig 18 & Fig 19) . Similar shifts can be seen in RBBR also where untreated dye shows peak at 1.915,2.236 & 3.106 min. After fungal degradation new peaks can be observed at 5.173,6.248,9.9 & 11.47 &12.449 minutes (Fig 20 & Fig 21). Differences in HPLC profiles of dye before and after fungal degradation show formation of new metabolites

after dye breakdown. Dhahir *et al* (2014) observed similar peak shift in HPLC profile during photo-oxidation of Rhodamine B. Similar kind of results were observed in previous studies (Dhanve *et al*., 2009; Gowindwar *et al*., 2014) where variations were found in the HPLC profile and FTIR spectrum of control dye and dye degraded by *Galactomyces geotrichum* .In a 2022 study by Hafidz *et al*, FTIR spectral analysis of methyl red dye degradation by *G. lucidum* depicted some changes in functional groups suggesting biodegradation activity. Saravanan *et al* (2022) estabilished breakdown of Rhodamine-B by Brevundimonas diminuta through FT-IR spectral scan & GC-MS analysis.

3.5 Phytotoxicity assessment

This was done by comparing the percentage germination of *P.mungo* seeds when soaked with treated and untreated dye solution.50 seeds were taken in petri dish and germination percentage was taken after 4 days. It was seen that compared to 28 % of germination in seeds soaked in aqueous solution of dye mixture, the treated ones showed 79% of seeds germination.

4. CONCLUSION

This work evaluated the decolorization& detoxification of Rhodamine and Remazol Brilliant Blue R by *Lentinus edodes*. ligninolytic enzyme laccase was found to be the major enzyme involved in breakdown of the industrial dyes. Toxicity of the degraded products was analysed on germination percentage of *P.mungo*. This study shows that *Lentinus* can be effectively employed for degradation of dyes and with proper optimization textile effluents can be treated with it. It's an ecofriendly and simple, method which can be employed for degradation of several synthetic dyes and other recalcitrant pollutants. Further studies can be conducted on effect of immobilization of fungal mycelium and the ligninolytic enzymes on the dye degradation process.

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