

Original Research

# Lignin Degrading System of *Phanerochaete chrysosporium* and its Exploitation for Degradation of Synthetic Dyes Wastewater

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## Abstract

Azo dyes are the main group of dyes used in different industrial applications. These dyes are highly toxic for aquatic life, so their removal is of utmost importance before they can be disposed of in a main water body. The present study focused on degrading/mineralizing the synthetic reactive dye wastewater. Initial experiments were done with four indigenous white rot fungi. *P. chrysosporium* (PC) showed more potential toward degradation of synthetic dye wastewater than other three fungal strains, so it was selected for further optimization of different fermentation parameters. Maximum decolorization (84.8%) of reactive dye wastewater was obtained at pH 5, inoculum size 4 mL, and 30°C. After optimizing experimental parameters, the effects of different nutritional factors like carbon and nitrogen sources were also studied. Decolorization of synthetic dye wastewater was increased from 84.8 to 89.2%, when rice bran was used as an additional carbon source. However, no increase in decolorization of synthetic dye wastewater was observed in the presence of nitrogen supplements. The screened fungal strain decolorized the wastewater up to 90%. The effect of different nutritional factors enhanced the degradation capability of the fungal strain under study. UV-visible and FTIR analyses confirmed the degradation of synthetic dye wastewater into simpler, non-toxic products.

**Keywords:** synthetic dye wastewater, *Phanerochaete chrysosporium*, mineralization, ligninolytic enzymes, effect of amendments, FTIR analysis

## Introduction

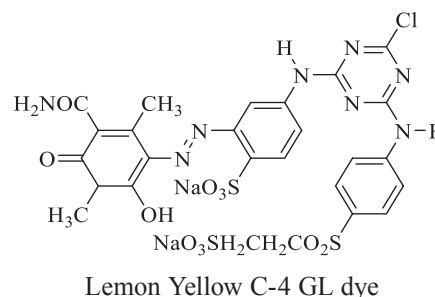
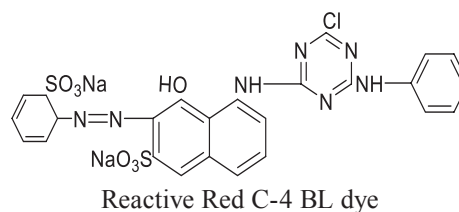
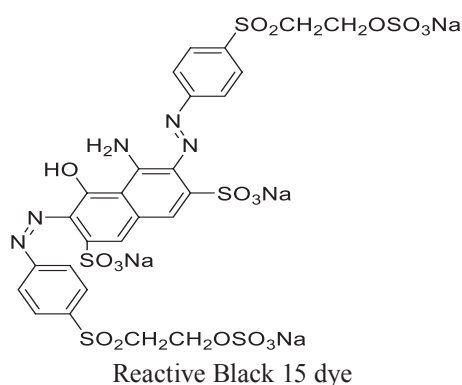
Azo dyes comprise almost one-half of all synthetic dyes, find their applications in different industries like food, paper, textiles, leather, cosmetics, etc. Among them, the textile industry is the main consumer of these dyes in its different operations [1]. About 15-20% of the dyes used in textile industries remain unutilized and are discharged into water bodies without any prior treatment. Dyes may significantly affect photosynthetic activity and have a significant impact on human health, as well as other animals [2]. These dyes are stable toward light, so they tend to reside in environments for a long period of time, causing a serious threat to the environment [3]. The complete removal of harmful dyes is a major concern [4]. Different physical methods are in use, but they are not efficient as they convert pollution from one phase to another [5]. Although chemical processes are found to be more efficient than physical ones, they are either costlier or they may generate byproducts that are more toxic than their precursors. On the other hand, biological treatment using bacteria or fungi are striking choices as they could be economically viable, easy to use, and environmentally approachable [6]. Different contaminants could be efficiently mineralized using microbes [7]. A unique characteristic of fungi is their ability to produce several non-specific enzymes. These non-specific extracellular and/or exoenzymes enable aquatic fungi to attack structurally diverse organic compounds that correspond to different pollutant classes. Hence, these fungi may serve as a new resource to treat wastewater [8]. The present study is focused on the screening of four different strains of white rot fungi for the degradation of synthetic wastewater having notorious dyes. The screened fungal strain was used for subsequent studies.

## Materials and Methods

### Chemicals

All chemicals were purchased from a chemical store and were of analytical grade.

Reactive dyes were kindly provided by Sandal Dyestuff, Pvt. Ltd. Dye structures are given below:



### Preparing Synthetic Dye Wastewater

Dye wastewater was prepared by mixing three reactive dyes (Reactive Blue, 140 mg/L; Reactive V violet, 152 mg/L; and Reactive Yellow, 1212 mg/L), hydrolysed starch (14 mg/L), sodium sulphate (28 mg/L), and sodium hydrogen phosphate (28 mg/L) in de-ionized water. pH of the sample material was set to 12 using 1M NaOH/0.5 M H<sub>2</sub>SO<sub>4</sub>. The reaction material was placed on a magnetic stirrer at 80°C for 90 minutes [4].

### Microorganisms

The pure culture of four white rot fungi (WRF) *P. ostreatus* (PO), *P. chrysosporium* (PC), *T. versicolor* (TV), and *G. lucidum* (GL) were taken from an industrial biotechnology lab, UAF, and grown in potato dextrose agar (PDA) media slants (pH 4.5, temperature 30°C) for one week. The slants were preserved at 4°C in a refrigerator [9].

### Preparing Inoculum

For preparing inocula, the pH of triplicate flasks having Kirk's nutrient medium was set to 4.5 with 0.1 M NaOH/0.05 M H<sub>2</sub>SO<sub>4</sub>. The flasks were placed in an autoclave at 121°C for 15 min. Fungal cultures from respective fungal slants were added in flasks. The flasks were placed in an incubator (120 rpm) at 30°C for 5-7 days to get homogeneous spores [10].

### Experimental Protocol

The flasks having 100 mL of dye wastewater and 100 mL of Kirk's medium (pH 4.5) were autoclaved for 15 min. at 121°C. The flasks were cooled and inoculated with 5 mL spore suspension of each fungus. The flasks were incubated at 30°C for 5 days. Supernatants were collected after centrifuging (1200 rpm for 10 mins) and absorbance was measured through a spectrophotometer

at  $\lambda_{\max}$  of 600 nm. The results were recorded as the average of triplicates.

### Optimizing the Biodegradation Process

Different environmental factors affect the microbial growth and their production [11]. Important growth-related factors (conc. of synthetic dyes wastewater 0.01-0.05%; pH 3-5; incubation temperature 20-40°C; inoculation size 1-5 mL) were studied. One factor at a time was varied while others were kept constant.

### Effect of Amendments

After optimizing experimental parameters, the effects of different carbon sources (glucose, starch, glycerol, wheat bran, rice bran) and nitrogen sources (yeast extract, maize gluten 30%, maize gluten 60%, corn steep liquor, ammonium oxalate) was studied to determine their effects on the production of fungal enzymes and level of dye decolorizing capability of fungus under study.

### Decolorization Assay Via UV-Vis Spectroscopy

The decolorization (%) efficacy was evaluated by measuring absorbance use of a UV/Visible spectrophotometer at 600 nm. The following formula was used:

$$\text{Decolourization (\%)} = (I - F) / I \times 100$$

I = initial absorbance

F = Absorbance of decolorized medium

### Enzyme Study

#### *Lignin Peroxidase (LiP) Assay*

1 mL of tartarate buffer (1 mM) of pH 3 and 1 mL of veratryl alcohol (1 mM) were added to 100  $\mu$ L of supernatant of treated wastewater. 500  $\mu$ L of  $\text{H}_2\text{O}_2$  (0.2 mM) was added in the reaction mixture. The reaction progress was determined by measuring absorbance at 310 nm [12].

#### *Manganese Peroxidase (MnP) Assay*

1 mL of sodium malonate (50 mM) buffer of pH 4.5 and 1 mL of  $\text{MnSO}_4$  (1 mM) were added in 100  $\mu$ L of supernatant of treated wastewater. 500  $\mu$ L of  $\text{H}_2\text{O}_2$  (0.1 mM) was added to the reaction mixture. The reaction progress was determined by measuring absorbance at 270 nm [13].

#### *Laccase Assay*

1 mL of malonate buffer (50 mM) or pH 4.5 and 1 mL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (1 mM) were added in 100  $\mu$ L

of supernatant of treated wastewater. 500  $\mu$ L of  $\text{H}_2\text{O}_2$  (0.2 mM) was added to the reaction mixture. The reaction progress was determined by measuring absorbance at 436 nm [14].

### *Spectral Studies*

The dye decolorization products were monitored by measuring the change in UV-vis spectra (from 200 to 800 nm) by means of a UV-vis spectrophotometer (Agilent 8453), whereas degradation investigation was checked by FTIR [15].

## Results and Discussion

### Screening White Rot Fungi (WRF) for Decolorization of Synthetic Dye Wastewater

Four strains of white rot fungi i.e. *P. ostreatus* (PO), *P. chrysosporium* (PC), *T. versicolor* (TV), and *G. lucidum* (GL) were initially screened. As the time was increased, the increase in decolorization of synthetic dye wastewater was observed by all fungal strains under study. Maximum decolorization was observed on the 6<sup>th</sup> day of incubation (Table 1). Among all fungal strains, *P. chrysosporium* (PC) showed maximum decolorization (82.9%) of dye wastewater; therefore, *P. chrysosporium* (PC) was nominated as a superlative applicant for further investigational work. Though all lignin-cellulosic enzyme systems were found to be responsible in the decolorization of dye wastewater by all fungal species, lignin peroxidase (LiP) was found to be the main enzyme more than manganese peroxidase (MnP) and laccase on the 6<sup>th</sup> day of nurturing (Table 1). Previous work reported that white-rot fungi are the most active biodegradables of lignocellulosic materials than other microbes [9]. In a previous study, lignin in a tobacco stalk was degraded up to 53.75% by *Phanerochaete chrysosporium* in a period of 15 days. The main enzyme responsible for degradation was found to be laccase [16]. Previous studies assessed that ligninolytic enzymes like laccases and peroxidases cause lignin degradation via free radicals like OH, which depolymerizes the phenolic and non-phenolic portions of lignin polymer, hence it mineralizes the whole lignin molecule [17].

### Effect of Synthetic Dye Wastewater Concentration

Dye molecules act as a substrate for microbes up to a certain concentration. A high concentration of dye molecules may cause a decline in growth of microbes, as it may act as growth inhibitor [4]. When synthetic dye wastewater concentration was increased from 0.01-0.05%, the percent decolorization gradually decreased. Lignin peroxidase enzyme was found

Table 1. Screening of WRF cultures for decolorizing synthetic dye wastewater

WRF	Decolorization (%) $\pm$ S.E				Enzyme activity (U/mL) $\pm$ S.E			Cell dry wt. (g)
	Day-2	Day-4	Day-6	Day-8	Lac	MnP	LiP	
PC	49.7 $\pm$ 0.51	62.1 $\pm$ 0.5	82.9 $\pm$ 0.54	60.1 $\pm$ 0.5	23.3 $\pm$ 0.55	33.3 $\pm$ 0.5	157.5 $\pm$ 0.5	1.09
PO	40.2 $\pm$ 0.52	53.2 $\pm$ 0.5	65.9 $\pm$ 0.52	44.3 $\pm$ 0.5	25.1 $\pm$ 0.55	73.9 $\pm$ 0.5	24.5 $\pm$ 0.5	0.12
TV	20.8 $\pm$ 0.52	33.8 $\pm$ 0.5	42.6 $\pm$ 0.51	51.6 $\pm$ 0.5	20.4 $\pm$ 0.5	101.9 $\pm$ 0.5	95.2 $\pm$ 0.53	0.06
GL	16.5 $\pm$ 0.53	31.8 $\pm$ 0.5	38.9 $\pm$ 0.53	46.1 $\pm$ 0.5	15.1 $\pm$ 0.5	99.7 $\pm$ 0.52	92.5 $\pm$ 0.5	0.04

Table 2. Effect of concentration of synthetic dye wastewater on its decolorization (%) and activities of ligninolytic enzymes of *P. chrysosporium*.

Conc. (%) $\pm$ S.E	Decolourization (%) $\pm$ S.E	Laccase (U/mL) $\pm$ S.E	MnP (U/mL) $\pm$ S.E	LiP (U/mL) $\pm$ S.E
0.01	80.93 $\pm$ 1.22	14.9 $\pm$ 0.41	9.1 $\pm$ 0.33	83.3 $\pm$ 0.48
0.02	55.675 $\pm$ 1.23	13.2 $\pm$ 0.42	8.5 $\pm$ 0.36	72.21 $\pm$ 0.47
0.03	42.687 $\pm$ 1.24	12.5 $\pm$ 0.46	7 $\pm$ 0.32	64.53 $\pm$ 0.46
0.04	32.448 $\pm$ 1.24	11.4 $\pm$ 0.45	6.1 $\pm$ 0.35	52.76 $\pm$ 0.47
0.05	24.705 $\pm$ 1.23	9.5 $\pm$ 0.45	5.1 $\pm$ 0.31	43.21 $\pm$ 0.46

to be more than laccase and manganese peroxidase enzymes (Table 2). Previous studies have shown that color elimination depends on the demolition of the chromophoric group. The ligninolytic system of the fungus attacks one dye molecule numerous times; a lesser quantity of the dye helps in the breaking of dye molecule while a larger quantity of the dye may cause a slower rate of dye deletion [18]. The dye molecule possesses  $-\text{SO}_3\text{H}$  groups, which may hinder the growth of microbes at higher concentrations [19]. Initial dye concentrations usually range from 50-1000 mg/L. Decolorizations mainly depend on the nature of the microbe and type of dye in use [20].

#### Effect of pH on Decolourization of Synthetic Dye Wastewater

pH is one of the most important parameters affecting the growth of microbes. Microbes can grow

in certain series of pH that favor nourishment and replication [21]. In general, fungi prefer to survive in acidic conditions, while bacteria breed in a neutral to alkaline atmosphere [22]. The effect of different pH (3.5-5.5) values on the decolorization (%) of synthetic dye wastewater was examined. As pH increased from 3.5-4.5, decolourization (%) of synthetic dye wastewater was amplified from 41.60% to 83.07%, but further increases in pH (5.5) resulted in a decrease in decolorization (46.5%) (Table 3). Similarly, production of lignin-cellulosic enzymes (LiP, MnP, Laccase) was maximum at pH 4.5; however, production of LiP was found to be greater (135.3 U/mL) than the other two enzymes. After inoculation, the maximal decolorizing ability by white rot fungi was seen in acidic conditions. The rate of color deletion appeared to decline quickly under strongly acidic or alkaline situations, regardless of dye structure [23].

Table 3. Effect of pH on decolorization (%) of synthetic dye wastewater and activities of ligninolytic enzymes of *P. chrysosporium*.

pH	Decolourization (%) $\pm$ S.E	Laccase (U/mL) $\pm$ S.E	MnP (U/mL) $\pm$ S.E	LiP (U/mL) $\pm$ S.E
3.5	41.63 $\pm$ 1.29	11.42 $\pm$ 1.29	10.59 $\pm$ 1.23	34.09 $\pm$ 1.33
4.0	57.35 $\pm$ 1.23	13.22 $\pm$ 1.23	12.54 $\pm$ 1.24	65.6 $\pm$ 1.34
4.5	83.07 $\pm$ 1.29	16.50 $\pm$ 1.26	14.04 $\pm$ 1.23	135.3 $\pm$ 1.35
5.0	61.3 $\pm$ 1.23	14.33 $\pm$ 1.25	13.86 $\pm$ 1.29	72.36 $\pm$ 1.29
5.5	46.5 $\pm$ 1.23	8.28 $\pm$ 1.25	10.10 $\pm$ 1.23	46.77 $\pm$ 1.29

Table 4. Effect of inoculum size on decolorizing (%) synthetic dye wastewater and activities of ligninolytic enzymes of *P. chrysosporium*.

Inoculum size (mL)	Decolourization (%)±S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
1	28.25±1.31	11.11±1.61	10.9±1.51	81.72±1.51
2	38.06±1.31	14.03±1.62	12.15±1.50	90.32±1.50
3	44.17±1.33	17.22±1.62	15.54±1.49	101.22±1.54
4	85.8±1.32	14.32±1.63	18.65±1.52	138.23±1.49
5	45.16±1.31	10.89±1.64	16.49±1.53	90.28±1.52

Table 5. Effect of temperature on decolorizing (%) synthetic dye wastewater and activities of ligninolytic enzymes of *P. chrysosporium*.

Temperature (°C)	Decolourization (%)±S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
25	43.6±1.18	4.12±1.43	3.34±1.47	45.32±1.46
30	84.8±1.19	7.11±1.44	8.11±1.46	134.32±1.46
35	47.0±1.20	5.81±1.46	6.81±1.48	48.92±1.47
40	38.47±1.21	4.23±1.47	4.11±1.47	32.47±1.48

### Effect of Inoculum Size

The effect of inoculum size was observed by keeping other parameters constant (pH 4.5, 30°C). As inoculum size increased (%), decolourization also increased, reaching a maximum value of 75.8% at inoculum size 4 and further increasing in inoculum size, decreasing its value to 45.16% (Table 4). A variation was noticed with the growth of different enzymes at the same value of inoculum size. LiP showed maximum production 103.23 (U/mL), while other enzymes like MnP and Laccase showed 18.68 (U/mL) 14.32 (U/mL) at inoculum size 4 mL. Further increases in inoculum size brought a decline in percentage decolourization of 46.16% as well as enzyme growth reducing to 10.89 (U/mL), 16.49 (U/mL), and 90.28 by laccase, MnP, and LiP. Most of the dyes can be degraded by liter-decomposing fungi. Their ligninolytic enzymes are involved in the degradation of textile wastewater having dyes. 4-5 mL of inoculum size is sufficient for maximum degradation of the textile wastewater with dyes [24, 25].

### Effect of Temperature on Decolourization of Synthetic Dyes Wastewater

Temperature is a very important parameter that needs to be measured, as it has a major effect on microbial growth and their dye decolorizing ability as well. Microbes can grow over a range of temperature; however, there is an optimum temperature for maximum proliferation of microbes under study [26]. As temperature was increased from 25°C to 30°C,

the decolorization (%) of synthetic dye wastewater increased from 43.6% to 84.8%. A further increase in temperature caused a decline in decolorization (%) of dye wastewater under study (Table 5). A similar trend was observed in production of enzymes by *P. chrysosporium* (PC). Lip production was maximum (134.32 U/mL) at optimized temperature (30°C) than MnP and laccase. Different fungi have different optimal growth temperatures. Most of them grow at 25°C, 30°C, or 35°C [27]. The optimal temperatures for enzymatic responses are typically higher, but the enzyme becomes thermally unstable at higher temperatures [3]. The optimal temperature may vary from microbe to microbe [4].

### Effect of Additional Carbon Sources

Carbon is essential for microbial growth, and to offer the source for oxidants the microbe needs to degrade contaminants. Glucose, fructose, sucrose, etc. have been used in most investigations while also starch and xylan look to be valuable [28]. Various carbon sources like starch, glycerol, glucose, rice bran, and wheat bran were studied as an additional carbon source. Each additional carbon source showed a positive input toward decolorization of dye wastewater under study. Maximum decolorization (89.2%) of synthetic textile dye wastewater by *P. chrysosporium* was obtained when rice bran was used as an additional carbon source to supplement other carbon sources. Production of LiP (146.81 U/mL) by *P. chrysosporium* was maximum when rice bran was added as an additional carbon source (Table 6). Other studies have observed the

Table 6. Effect of additional carbon sources on decolorizing (%) synthetic dye wastewater and activities of ligninolytic enzymes of *P. chrysosporium*.

Carbon sources	Decolourization (%)± S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
Starch	41.1±1.51	15.89±1.48	8.34±1.50	94.93±1.47
Glycerol	44.7±1.52	16.67±1.45	8.07±1.49	94.63±1.46
Glucose	48.4±1.53	17.36±1.48	8.32±1.53	98.92±1.48
Rice bran	89.2±1.50	18.25±1.45	11.1±1.52	146.81±1.43
Wheat bran	65.1±1.51	16.20±1.48	10.24±1.51	131.62±1.42

Table 7. Effect of additional nitrogen sources on decolorizing (%) synthetic dye wastewater and activities of ligninolytic enzymes of *P. chrysosporium*.

Nitrogen Sources	Decolourization (%) ± S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
Corn steep liquor	53.23±1.52	5.00±1.52	3.73±1.42	30.1±1.52
Yeast extract	55.91±1.51	7.36±1.52	4.65±1.41	33.3±1.53
Maize gluten 60%	63.45±1.55	8.4±1.53	5.99±1.40	50.3±1.55
Ammonium oxalate	80.08±1.55	9.0±1.51	6.77±1.42	75.7±1.55
Maize gluten 30%	64.08±1.55	6.47±1.52	5.30±1.45	38.5±1.56

effect of carbon sources on dye decolourization to optimize the yields of decolourization [29]. Researchers reported that decolourization is more effective in supplementing carbon sources [30].

#### Effect of Additional Nitrogen Sources

Various nitrogen sources were introduced to investigate the percentage decolorization of synthetic textile dye wastewater by *P. chrysosporium* (PC). The need of nitrogen for proliferation and particularly enzyme production varies distinctly between fungal cultures. It is well acknowledged that the production of lignin-cellulosic enzymes is more in nitrogen starvation [24], but a maximum 80.08% wastewater was decolorized when ammonium oxalate was used as a nitrogen source. Minimum 53.23% decolourization was shown in the presence of corn steep liquor (Table 7). Researchers reported that Remazol black dye decolorization was markedly decreased in the presence of nitrogen sources like peptone, yeast extract, etc. [31], which might be because microbes could efficiently use dye carbon for their respiration and dye nitrogen to build their body proteins [32].

#### Spectral Analysis

UV-visible spectrum of unprocessed synthetic dye wastewater displayed a peak in the visible region ( $\lambda_{max} = 600$  nm). The treated synthetic dye wastewater showed a reduction in the height of spectral peak depicting absorption maxima, which confirmed

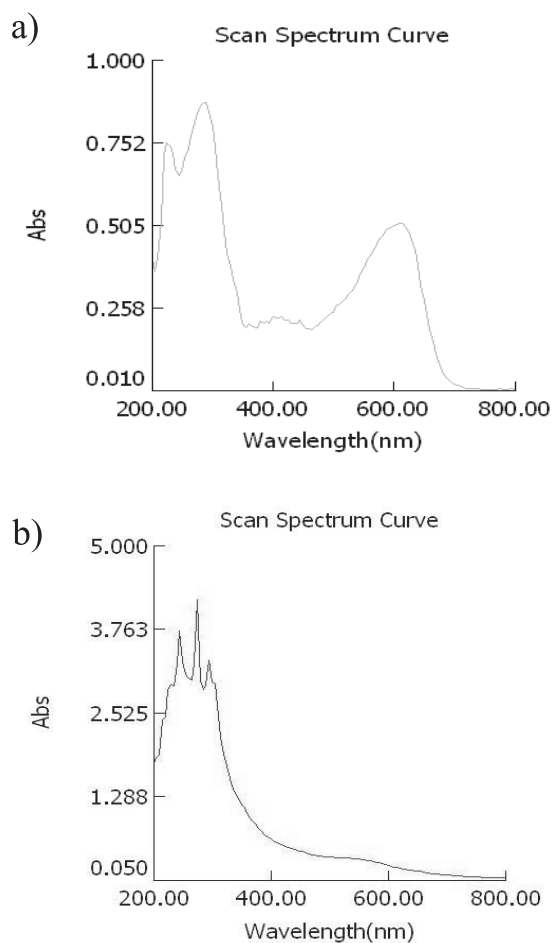


Fig. 1. UV-visible spectrum of synthetic dye wastewater before a) and after b) biological treatment by *P. chrysosporium*.

the decolorization of dye wastewater under study. The appearance of spectral peak in the UV-region confirmed the degradation of synthetic dye wastewater under study (Fig. 1a-b). FTIR analysis was done to describe the metabolites of dye wastewater under study. FTIR spectrum of unprocessed synthetic dye wastewater exhibited peaks at 3344.59, 2352.21, and 1646.3  $\text{cm}^{-1}$  for  $-\text{OH}$  stretch of phenol,  $\text{N}=\text{N}$  stretch, and  $\text{C}=\text{C}$  stretching of monosubstituted benzene ring (Fig. 2a), while FTIR analysis of the treated wastewater showed peaks at 3313.55  $\text{cm}^{-1}$  for  $-\text{OH}$  stretch, 2945.51  $\text{cm}^{-1}$  and 2834.66  $\text{cm}^{-1}$  for  $\text{C}-\text{H}$  stretching of alkyl benzene, 1460.07  $\text{cm}^{-1}$  for  $-\text{C}-\text{H}$  bending, 1127.51 and 1025.52  $\text{cm}^{-1}$  for  $\text{C}-\text{O}$  stretch,

and 613.15  $\text{cm}^{-1}$  for monosubstituted benzene ring (Fig. 2b). The appearance of peak at 3313.55  $\text{cm}^{-1}$  indicated the formation of carboxylated products. The nonappearance of peaks amid 3400 to 3380  $\text{cm}^{-1}$  for  $-\text{NH}$  stretch showed that no aliphatic and aromatic amines were produced by biological treatment. The peak distinctive of azo bond at 1646.3  $\text{cm}^{-1}$  of dye was not present in the treated sample, indicating the degradation of dye wastewater to aromatic amines as midway products, which were further oxidized to simplified products. Researchers reported that using the fungal strain decolorization of dyes with the wavelength range 250-700 nm could be degraded to simpler products [4, 33].

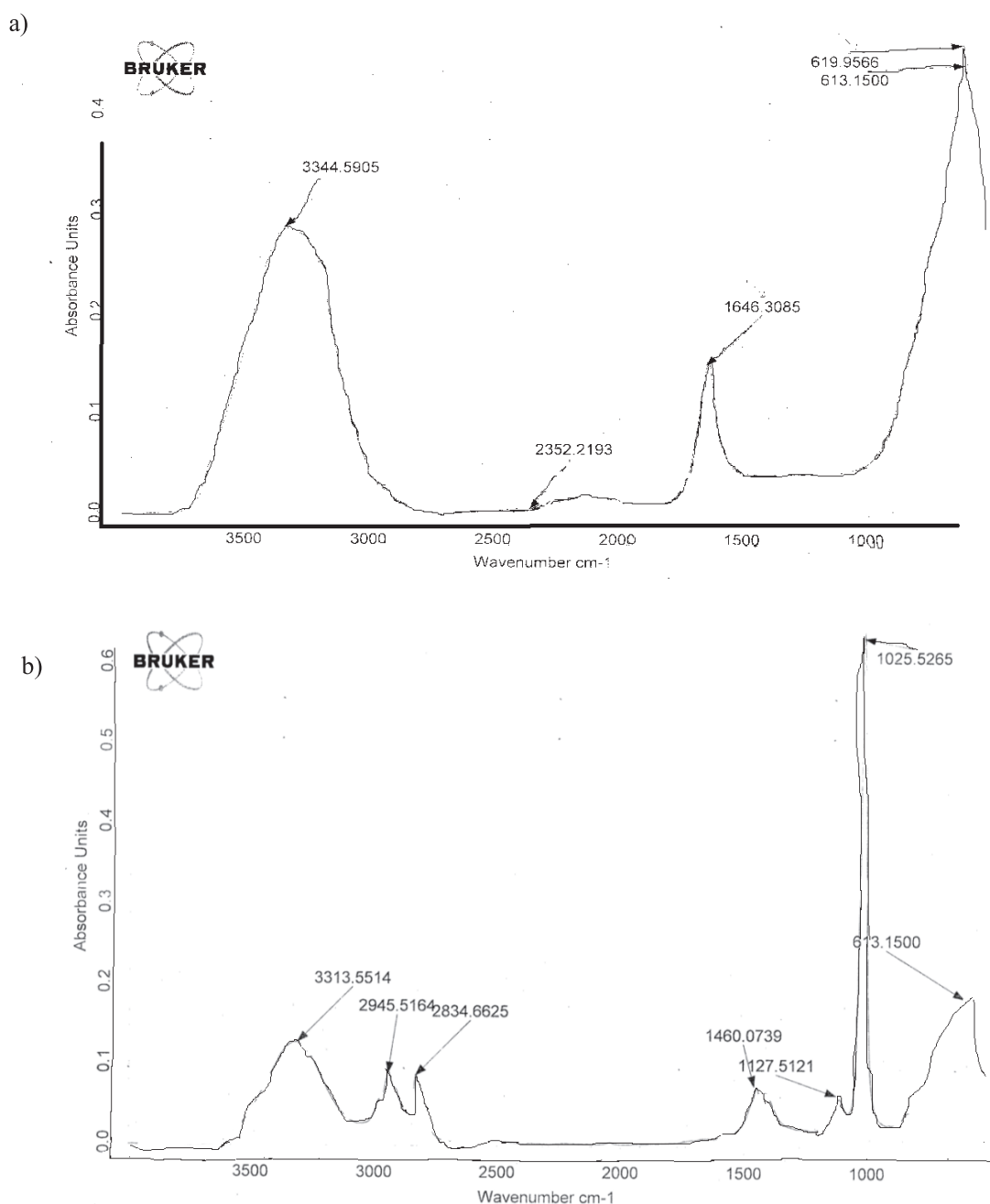


Fig. 2. FTIR spectrum of synthetic dye wastewater before a) and after b) biological treatment by *P. chrysosporium*.

## Conclusions

Biodegradation was found to be a more efficient technology in terms of its eco-friendly nature and complete degradation of almost all dangerous dyes. *Phanerochaete chrysosporium* was found to be the best candidate for detoxifying the synthetic dye wastewater under study. Lignin peroxidase of *Phanerochaete chrysosporium* was found to be responsible for detoxifying the wastewater under study. It can be concluded that *Phanerochaete chrysosporium* could be the best candidate for detoxification/mineralization of toxic dyes.

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## Conflict of Interest

All authors declare no conflict of interest.

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