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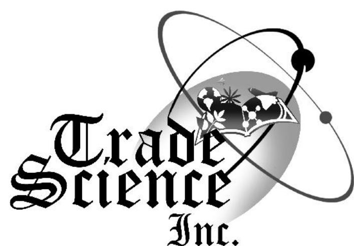

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Application of ligninolytic fungus in decolourisation and degradation of some textile dyes

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ABSTRACT

Plentiful production of the synthetic dyes is directly interrelated with the soil and waterpollution. Therefore,to overcome this problem and its mitigation is always been serious and important issue worldwide. Application of microbial enzymes to remediate these recalcitrant compounds from the environment is very well-knownbiological solution, which is effective since last few decades. Present study deals with application of ligninolytic enzymes produced by a wild strain of *Phanerochaetechrysosporium*to degrade four reactive textile dyes*viz*. Reactive Red HE8B, Reactive Black B, Reactive Golden Yellow HR, and Reactive Violet 5R. The fungus was obtained from the decaying wood log of the tree growing Junagadh Forest of Gujarat State.It is not only proved to be potential strain indecolourisationofdyes but also proficiently degraded all the dyes under solid and liquid decolourisation media. The decolourisation and degradation study was efficiently supported by the FTIR analysis of dyes treated with ligninolytic enzymes produced by solid state fermentation.On plate ddecolourisation of dyes initiated after threedays of fungal inoculation and the petri plates with 10 mg/L concentration of all four dyes were completely decolourised within 11 days, except Reactive Red HE8B which took 13 days after incubation. Supplementation of different sources of carbon and nitrogeninfluencedthe process of decolourisation, in which dextrose and asparagines were found to be excellent supporters respectively.Interestingly one of the nitrogen sources i.e. sodium nitrite inhibited growth of the fungus.Influence of inoculum size on decolourisation was performed with different inocula size on solid and in liquid media, where onetothreediscs (10mm diameter agar plugs) were reported to be more effective in the rate of the decolourisation.

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KEYWORDS

Phanerochaetechrysosporium;
Decolourisation;
Degradation;
FTIR;
Textile dyes.

INTRODUCTION

The Indian textile industry counts among the leading textile manufacturersin the world and is the second largest producer of textilesand garments[11] where Gujarat state is one of the textile hub and apparel zone in the western part of India. Synthetic dyes are considered to be the pillars for the textile industries, among which azo dyes are the largest group used by the dying and printing houses. They contain

the azo group (-N=N-) as the chromophores which makes them recalcitrant to the degradation and therefore exhibits the acute toxic effect on the flora and fauna. To mitigatethis challenge, severalphysico-chemical treatment technologies have been developedworld wide but unfortunately most of them are proved ineffective due to the chemical stability of these pollutants. Therefore,most effortsare now concentrating to the biological methods where microorganisms are used to meet the desired depollution.

Perusal of literature indicates that decolourisation and degradation of differentazo dyes has been studied thoroughly by using bacteria as the biological agent.Beside bacteria, application of white rot fungi has also received a considerable attention owing to the action of extracellular non-specific enzymes produced by them. The ligninolyticenzymes produced by white rot fungi have the potential to degrade complex compound such as lignin in their natural environment. This property of these enzymes can be efficiently applied in degradation of synthetic dyes which has similar chemical structures like lignin. Therefore, different white rot fungi are extensively studied for their application in bioremediation of textile dyes to counteract environmental pollution[12,23,24,25]. Among them, *P.chrysosporium* has been studied widely due to its vital role in the process of biodegradation[2,8,9,15,23,33]. The virtue of our study with the same fungus lies in its wild collection from the forest of Gujarat state (India) which has not been explored earlier. In our previous study, *P.chrysosporium* was investigated for their enzyme production through Solid state fermentation and biodegradation of reactive dyes. Main objective of the present study was to examinewhether:i) decolourisation of all four reactive dyes with different structures and λ_{max} occurs at the same rate in solid and liquid medium? ii) Inoculum size and supplementation of different carbon and nitrogen sources to growth media influence the process of decolourisations and degradation?iii) Dyes are decolourised or show any structural alteration after treating with the ligninolytic enzymes produced by*P. chrysosporium*?

MATERIAL AND METHODS

Isolation, purification and screening of the fungus

Several white rot basidiomycetesfungi were collected from the different forest of the Gujarat state. They were surface sterilized by routine method using 0.1% of $HgCl_2$ and 70% ethanol. The treated samples were inoculated on different growth media and incubated at room temperature (28-38° C). Isolated strains were purified by serial culture and maintained on optimized media i.e. Malt Extract Agar (MEA) at 4°C. All the purified strains were screened for production of ligninolyticenzymes, from which KSR17 was selected for the study and molecular identification was done with the help ofChromous Biotech Pvt. Ltd., Bangalore (India). The fungus was identified as *Phanerochaetechrysosporium* and the sequence was submitted to NCBI with accession number AB361645.

Chemicals and dyes

Malt extract powder and the sugars used in the study were obtained from Himedia (India). DMAB (3-dimethyl amino benzoic acid), MBTH (3-methyl-2-benzothioazolinone hydrazinehydrochloride), H_2O_2 and Manganese Sulphate ($MnSO_4$) were purchased from National chemicals Ltd., (India). The nitrogen sources were obtained from Qualigen (India). All four water soluble textile dyes (Reactive Red HE8B, Reactive Black B, Reactive Golden Yellow HR, and Reactive Violet 5R)used in the present study were obtained from the dying, printing and processing houses. Other chemicals acquired for the studies were of analytical grade.

Dye decolourisation

Decolourisation studies of four different reactive dyes were carried out in both the solid and liquid media. Solid plate decolourisation was performed by supplementing five different concentrations (10, 50, 100, 250 and 500 mg/L) of each dye along with the control after autoclaving MEAmedia (2%). Plates were inoculated centrally with 10mm diameter agar disc from the seven days old culture of *P. chrysosporium*. Decolourisation of the dyes

was monitored at the regular interval and zone of growth and decolourisation was measured.

Liquid decolourisation was performed in 150 mL Erlenmeyer flask each containing 25 mL of autoclaved Malt Extract (ME) broth (2%) supplemented with respective dye (10mg/L). All the flasks were inoculated with 3 disks of 10mm diameter agar plugs from actively growing culture and maintained at static condition in incubator at 27° C. The inoculated flasks were harvested at the regular interval and the filtrate was subjected to the spectrophotometric analysis at the maximum visible wavelength of absorbance (λ_{max}) for individual dyes in order to monitor the decolourisation. The percent decolourisation was calculated by following equation.

P% = A0 " A1 × 100

A0

Where, P% is the percentage decolourisation, A0 and A1 represent the initial and final concentrations of azodyes respectively.

All the experiments were performed in triplicates and average values were considered.

Influence of carbon and nitrogen sources on decolourisation

Five different carbon (dextrose, sucrose, fructose, maltose and lactose) and nitrogen (ammonium sulphate, sodium nitrite, asparagine, and urea) sources were added to the medium as the co-substrates at the concentration of 10 mg/L and checked for their effect on the decolourisation on both solid and liquid decolourisation. The influence of these substrates on solid plate decolourisation was conducted by inoculating the plates containing respective dye, media and carbon/nitrogen source as co-substrate with 10mm agar plugs from the actively growing culture. The zone of growth and decolourisation was measured at the regular interval and uninoculated plates were treated as controls. In case of liquid decolourisation 150 mL flasks containing media, respective dye and carbon/nitrogen sources (1%) were inoculated with 3 plugs of 10mm diameter from the *P.chrysosporium* plates and analysed through spectrophotometric analysis after every 3 days.

Influence of inoculum size on decolourisation

Petri plates containing MEA with the dyes were inoculated with the four different inoculum sizes (1 to 5, 10mm diameter agar plugs) and zone of growth and decolourisation were measured at the regular interval to find out the most appropriate inoculum size for the optimum decolourisation. However, the effect of inoculum size on liquid decolourisation was measured by inoculating the ME broth with different inoculum size (3, 6, 9, 12, and 15 of 10mm diameter agar plugs). All the flasks were harvested on the 5th day of inoculation and filtrate was subjected to the spectrophotometric analysis where the flasks with only medium without dye were considered as control and un-inoculated flasks with medium and respective dyes were considered as blank.

Enzyme production, partial purification and molecular weight determination

The potential of enzyme production by the fungus was carried out using the solid state fermentation technique where different agro-industrial wastes were used to optimize the substrate. Among different agro-industrial wastes used, wheat straw was found to be the best substrate;therefore, further studies were carried out by using wheat straw for the optimisation of inoculum size and incubation time. Enzyme assay was performed following the method described by Vyas et al.[3]. Partial purification of the crude enzyme was carried out by ammonium sulphate precipitation method as described by[26]. The molecular weight of the partially purified enzyme was evaluated by Electrophoresis method described by Laemmli[31]. Further details are as described earlier by Koyani et al.[25].

Biodegradation analysis by FTIR (Fourier transform infrared spectroscopy)

Degradation of the dyes using the partially purified enzyme was corroboratedby FTIR analysis of dyes treated with partially purified enzymes. For each dyes, concentration of 10 mg/Lwas prepared in 10mLand treated with 500µL of partially purified enzyme for 48 hours and then dried at the room temperature. The powder was processed byKBr pellet method (Mane et al.2008). The samples were

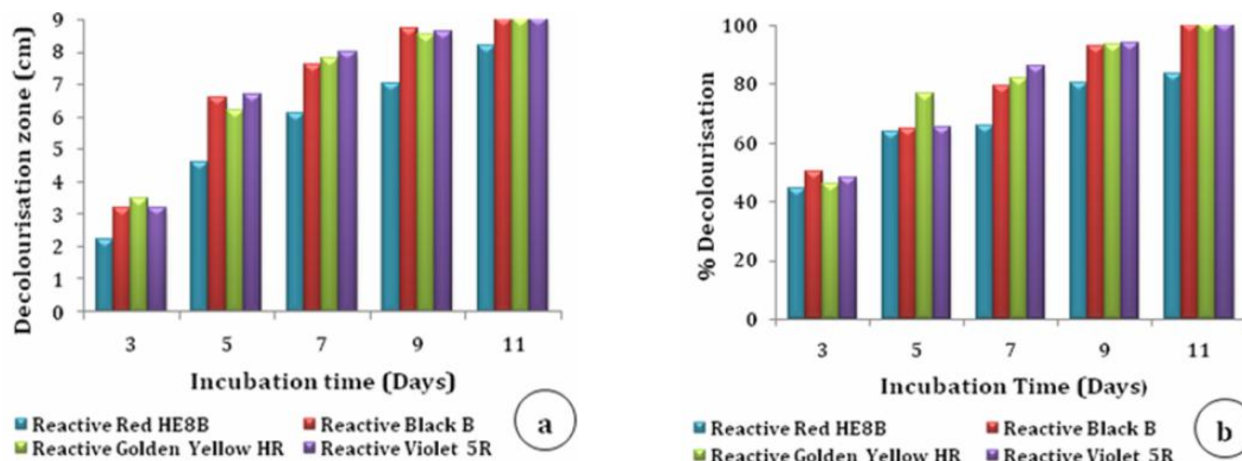

Figure 1: Decolourisation of the reactive textile dyes (10 mg/L) by *Phanerochaetechrysosporium* measured as decolourisation zone (a) as well as % decolourisation (b) at different time intervals

analysed with 30 scan speed at 10^{-4} resolution, in the mid region of 500- 4000 cm^{-1} by using Shimadzu 8400.

RESULTS AND DISCUSSION

Dye decolourisation

The magnitude of the research efforts initiated in the last decade on the degradation oftextile dyes reveals seriousness of the environmental problem caused due to reckless disposal of textile effluents released in the environment[24]. Various physico-chemical methods have been tried to overcome the problem of environment deterioration but recalcitrant compounds remained unresolved due to complex chemical structure. As an alternative, biological treatment of the textile dyes using white rot fungi is the best cost effective and environmental friendly approachover physical and chemical techniques. Therefore, several studies were carried out in search of potential fungus that can efficiently decolouriseand degrade different recalcitrant compounds. In the present study, *P.chrysosporium*a wild isolate from the decaying wood exploited and it is proved to be potential organism to degrade four reactive textile dyes (*viz.*Reactive Red HE8B, Reactive Black B, Reactive Golden Yellow HR, and Reactive Violet 5R). Ten mg/L concentration of each dye was completely degraded in both solid and liquid media in 11 days of fungal inoculation except Reactive red HE8B which took 13 days for complete decolourisation (Figures 1, 2a-h). Delay in decolourisation of Reactive red HE8B may be associated with its more recalcitrant nature due to chemical structure. Similar correlation about complexity of dye structures and decolourisation of synthetic dyes has also been established earlier stud-

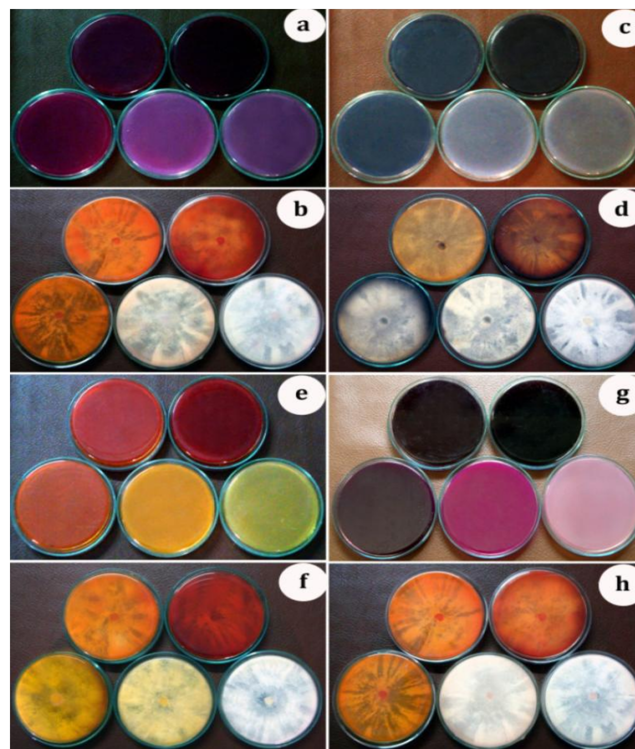

Figure 2 : On plate decolourisation of four reactive textile dyes i.e. Reactive Red HE8B (a: Control; b: Inoculated with *Phanerochaetechrysosporium*); Reactive Black B (c: Control; d: Inoculated with *P. chrysosporium*) Reactive Golden Yellow HR (e: Control; f: Inoculated with P. *chrysosporium*), Reactive Violet 5R (g: Control; h: Inoculated with *P. chrysosporium*), at five different concentrations (10, 50, 100, 250 and 500 mg/L- from right to left)

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ies[1,7]. Time required for the decolourisation also depends on the concentration of the dyes used in the[25]. All other four concentrations of the dyes tested in the present investigation were also been decolourised successfully but the time required for the same increased with the increase in concentration of dyes from 10, 50, 100, 250 and 500 mg/L (Figure 2). All dyes upto the concentration of 100mg/L were decolourised rapidly but thereafter it became slow and the concentration above 1 g/L was found to be inhibitory for the growth of the fungus and rate of decolourisation.

Influence of carbon and nitrogen sources on decolourisation

Available literature indicates that supplementation of growth media with various carbon and nitrogen sources boost the potential of enzyme production ability of fungus[16,17,19,20,21,] which is directly related with the rate of dye decolourisation and degradation. Therefore, in order to examine the influence of carbon and nitrogen sources on decolourisation,both liquid and solid media were supplemented with10 mg/L of five different carbon (Dextrose, Sucrose, Lactose, Maltose and Fructose) and nitrogen (Ammoniumsulphate, Urea, Asparagine, Sodium nitrate and Sodium nitrite) sources respectively. Among them,only dextrose and asparagine were found to be more proficient decolourising raiser as carbon and nitrogen sources respectively while others showed minor or undetectable variation in the rate of decolourisation. Carliellet al.[3] and Kapdanet al.[13] also reported that growth media supplemented with glucose enhanced the rate of decolourisation. Surprisingly, one of the nitrogen sources i.e. sodium nitrite was noted to be growth inhibitor (Figure 3).

There are no unanimous opinions about the role of nutrient supplements in the process of dye

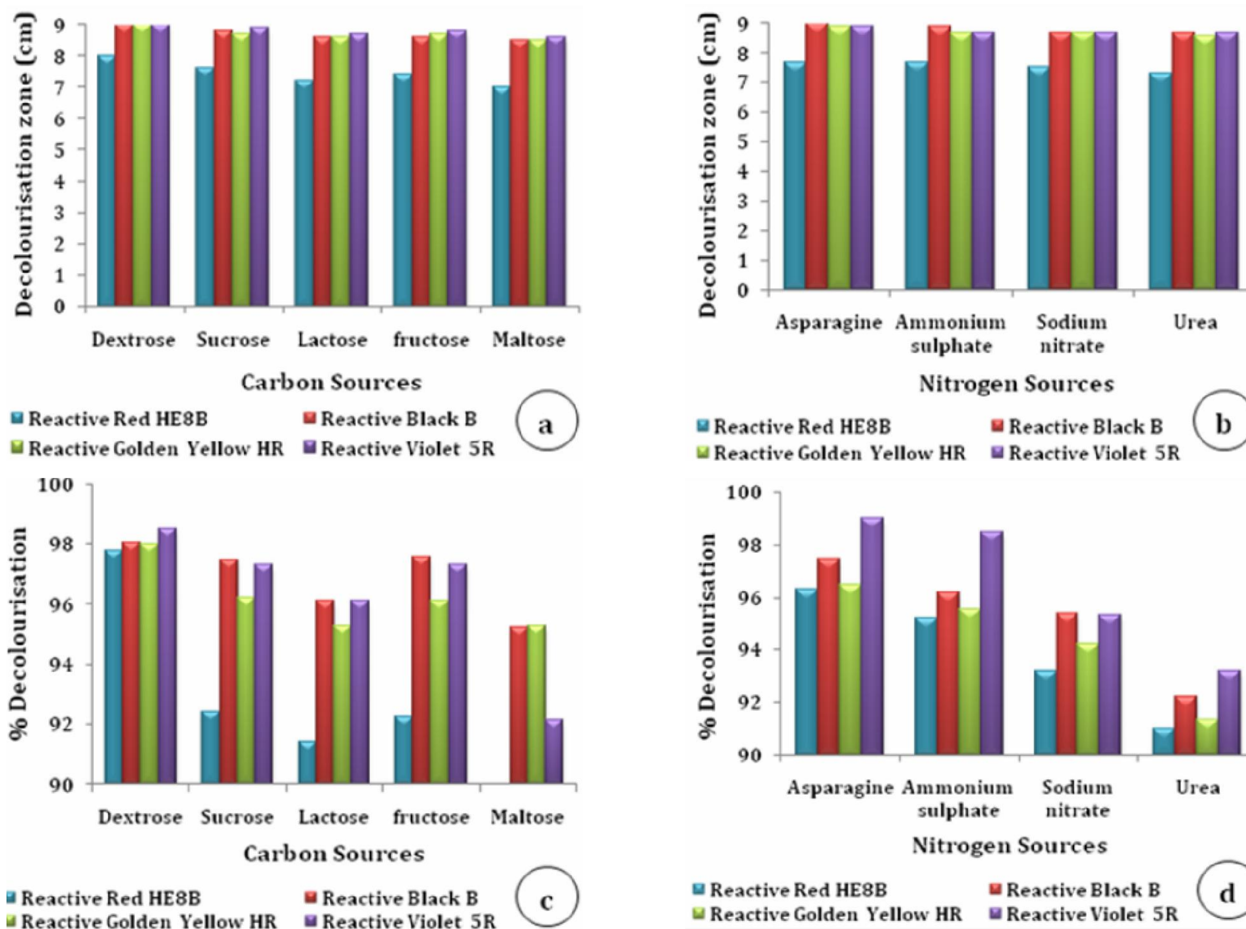

Figure 3 : Influence of carbon and nitrogen sources on solid plate (a and b) as well as liquid decolourisation (c and d) by *Phanerochaetechrysosporium*

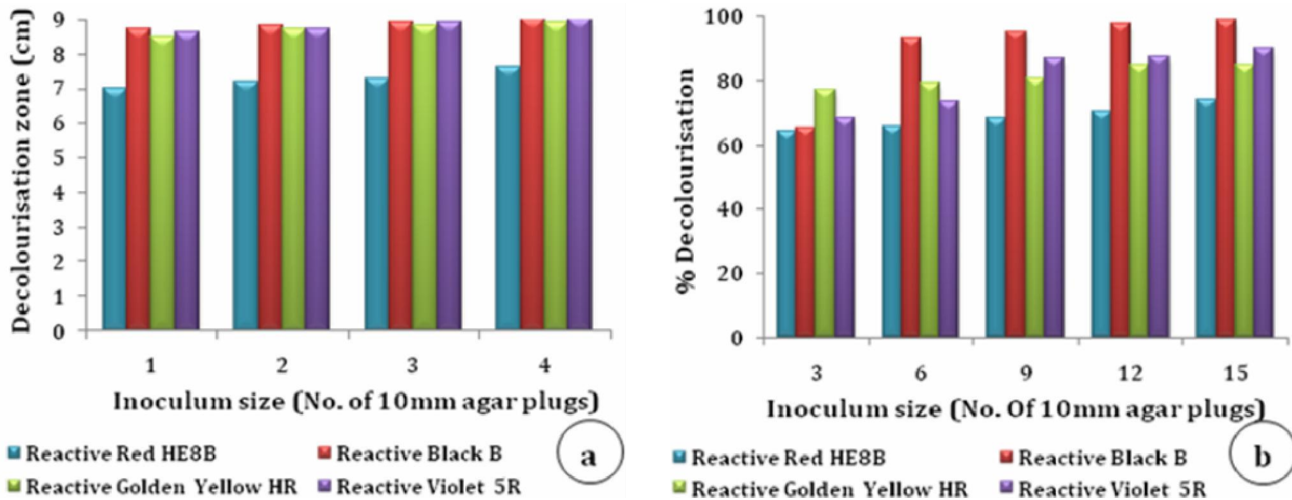

Figure 4 : Effect of inoculum size on solid plate (a) and liquid (b) decolourisation

Figure 5 : Comparison of the FTIR spectra of untreated and treated dyes with 500 µL of ligninolytic enzyme produced by *Phanerochaetechrysosporium*; a) Reactive Red HE8B b)Reactive Black B, c)Reactive Golden Yellow HR, and d) Reactive Violet 5R.

decolourisation. Available literature indicates that glucose was found to play important role in supporting decolourisation[3,13,30]. Similarly supplementation of nitrogen sources also enhancesthe production ligninolytic enzyme and ultimately the rate ofdye decolourisation[6]. On contrary to the present study, results obtained by[12] did not show any direct relationship between the carbon sources and decolourisation capacity of the strains. *Irpexlacteus* showed suppressed activity of enzymes in presence of nitrogen sources. On the other hand, Mester and Field[29], and Gianfreda et al. [17], noticed that some white rot fungi do not show any effect of carbon and nitrogen sources on ligninolytic enzyme production in the expected trend.

Effect of inoculum size on decolourisation

It is the general notion that larger size of inoculum shall fasten the rate of decolourisation. Therefore, the relationship between inoculum size and rate of decolourisation was investigated by inoculating 1 to 5 agar plugs (10mm diameter)per petri plate onthe solid decolourisation media whilefive different inocula size i.e. 3, 6, 9, 12, 15agar plugs (10mm diameter) per flask were inoculated inliquid media. Far away from our general perception that increase in the inoculum size could increase decolourisation rate but no such correlation was observed in the present study. One-three inoculum size was proved to be more efficient in solid and liquid decolourisation respectively whileno significant difference was observed in the rate of decolourisation when inocula size is being increased (Figure 4).

Enzyme production, partial purification and molecular weight determination

Solid state fermentation is always considered to be better methodology for the ligninolytic enzyme production as it mimics the natural habitat of the fungi[10]. Among all different solid substrates (i.e. wheat straw, saw dust, banana stem, rice straw, sugarcane bagasse, ground nut shells) used for the enzyme production, wheat straw was found to be more promising for the optimum production of Manganese Peroxidase, Manganese Independent Peroxidase and Laccasei.e. 487.9 IU/mL, 475.92 IU/mL,177.32 IU/mLrespectively[25]. Earlier studies have also proved Wheat straw as the better substrate for the ligninolytic enzyme[4,18,34]. Crude enzyme produced by *P.chrysosporium* was subjected to the partial purification through ammonium sulphate precipitation method at 4°C and up to 80% saturation. The maximum enzyme activity for manganese peroxidase (607.35 IU/mL), manganese independent peroxidase (539.27 IU/mL) and laccase (263.03 IU/mL) was exhibited in the 60% saturated fraction which was further experimented to the molecular weight determination. In the present investigation, ligninolytic enzyme produced by the *P.chrysosporium* was of 52.8kDa molecular weight[25]. which is slightly higher than the previous report (40kDa) by Tien and Kirk[22].

Biodegradation analysis by FTIR (Fourier transform infrared spectroscopy)

*Phanerochaetechrysosporium*not only decolourisedall the dyes testedbut it alsodisrupted thecomplex structure of dyes. It wasevident fromspectra obtained by Fourier Transform Infrared Spectroscopy analysis of treated and untreated dyes. Degradation of chemical structure of dyes directly entails the ligninolytic system of the white rot fungi. Different stretching vibrations expressed the existence of different groups present in the dyes while in degraded samples shifting of the peaks is evident in Figure 5.Previous literature has also been supported by the similar degradation studies and shifting of the peaks in the degraded samples[25,27,28].

CONCLUSION

The present investigation wild isolate of *P. chrysosporium* is revealed as potent strain to decolouriseand degrade four reactive textile dyes under solid and liquid media. Rate of dye decolourisation is influenced by the complexity of chemical structure and concentration of the dyes. Among the all treated dyes Reactive Red HE8B was found to be very complex, while increase in concentration of the dyes decreases the rate of decolourisation. Other parameters like carbon and nitrogen sources and inoculum size also influenced

the process of dye decolourisation. The role of ligninolytic enzymes in degradation of the dyes was confirmed by FTIR analysis of the samples treated with partially purified enzymes, whichshowed stretching vibrations in different chemical groups within different dyes investigated. The present study demonstrates that *P.chrysosporium* has potential not only to decolourise but also degrade the complex reactive textile dyes through its ligninolytic enzyme system. Further studies are warranted on the analysis of degraded dye products for their toxicity after treatment.

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