Role of Enzymatic System of Screened *Pleurots ostreatus* IBL-02 in the Bio-Removal of Synthetic Dyes Effluent

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Summary: White rot fungi have exclusive capacity to decolorize synthetic azo dyes. Five different fungal strains namely *S. commune* IBL-01 (SC), *P. ostreatus* IBL-02 (PO) *P. chrysosporium* IBL-03 (PC), *T. versicolor* IBL-04 (TV) and *G. lucidum* IBL-05 (GL) were used for the decolorization of synthetic dyes effluent. *P. ostreatus* IBL-02 showed maximum decolorization of synthetic textile effluent, so it was selected for the optimization of experimental factors. Different physico-chemical parameters were optimized using *Pleutorus ostreatus* IBL-02 (PO), for the maximum de-colorization of synthetic dyes effluent. Under optimum conditions, *P. ostreatus*IBL-02 (PO) decolorized the synthetic dyes effluent by 92.7%. Effect of various amendments like carbon and nitrogen sources on the decolorization and mineralization of synthetic dyes effluent by *Pleutorus ostreatus* IBL-02 was also studied. Ligninolytic enzymes at the end of each experiment were studied to find their role in decolorization and mineralization of synthetic dyes effluent. UV-Visible spectral analysis was used to indicate the decolourization of treated dyes effluent sample. A diminution in spectral line height displaying absorption maxima designated decolorization (%) while a move of spectral line to the UV-region depicted the degradation of synthetic dyes effluent.

Keywords: Synthetic dyes effluent; White rot fungi; Degradation; Ligninolytic enzymes; Process optimization; Spectral analysis

Introduction

In world ecosystem, toxicity is increased day by day due to the rapid growth of industry area. Synthetic dyes are commonly used in many industries. There are three groups of synthetic dyes such as azo dyes, phthalocyanine and anthraquinone. The 50% of total dyes are azo dye annually produced [1]. These synthetic dyes have complex and stable structure which cannot easily be degraded [2]. These dyes tends to reside there for a longer time due to their stable nature [3]. So, the efficient treatment is needed to remove these dyes from the environment. Decolorization of textile effluents trough physical and chemical technologies is commercially unattractive and very expensive as well. Biodegradation is a technique which is cheaper and eco-friendly in nature. It not produce huge quantity of sludge and is an alternative to other technologies [4]. The biodegradation of toxicants like xenobiotics, dyestuffs etc. is gaining more popularity [5]. Ligninolytic enzymatic system of white rot fungi is found to be effective in environmental remediation [6-9].

The present study was focused on screening of white rot fungi based on their potential for degradation of synthetic dyes wastewater. The experimental factors were than optimized using screened white rot fungal strain. The target dyes that used widely and well known to be problematic in terms of both treatability and toxicity were taken into consideration in the current study. The effectiveness of the biotreatment was assessed through spectral analyses.

Experimental

Chemicals

All chemicals were of analytical grade. Reactive dyes were purchased from local market.

Preparation of Synthetic effluent

Synthetic dyes effluent was prepared by mixing hydrolysed reactive dyes (Reactive Black 15 (139 mg/L); Reactive Yellow C-4 GL (150 mg/L); Reactive Red C-4 BL (1215 mg/L), hydrolysed starch (13.9 mg/L), Na_2SO_4 (27.8 mg/L) and Na_2HPO_4 (27.8 mg/L) in de-ionized water. Then it was placed on hot plate on magnetic stirrer at 80°C for 1.5 h after adjustment at pH 12.

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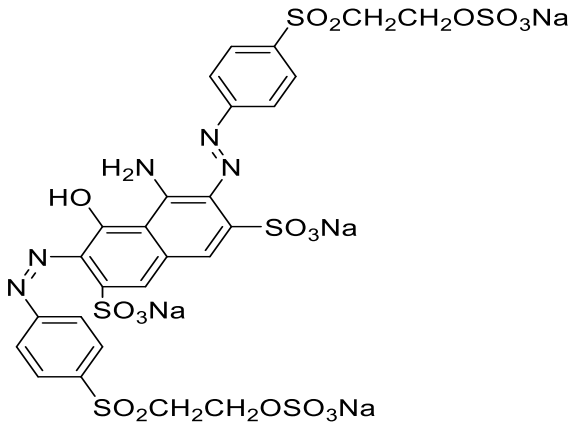
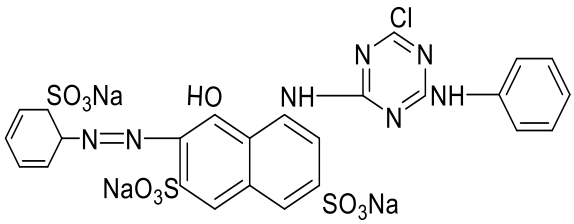
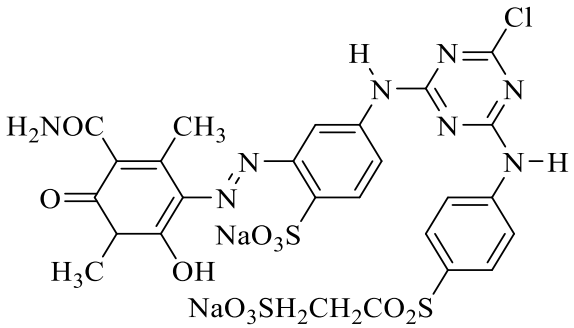


Reactive Black 15 dye



Reactive Red C4 BL dye



Lemon Yellow C4 GL dye

Fig. 1: Chemical structures of Dyes.

Microorganisms

Pure culture of five white rot fungi *S. commune* (SC), *P. ostreatus* (PO), *P. chrysosporium* (PC), *T. versicolor* (TV) and *G. lucidum* (GL) were obtained from Industrial Biotechnology Lab, UAF and grown on potato dextrose agar (PDA) medium slants for 6-7 days at pH 4.5 and temperature 30 °C. The slants were preserved at 4 °C in refrigerator.

Preparation of Inoculums

Inoculums were prepared for each experiment. The flasks containing Kirk's medium for individual fungi were adjusted at pH 4.5 with 01 M NaOH/0.5 M H_2SO_4 and autoclaved at 121°C for 15 mints. The flasks were inoculated with fungi spores from respected slants culture and placed in inoculators under shaking 120 rpm at 30 °C for five days to get homogenous spores suspension [10].

Experimental protocol

100 mL textile synthetic dye effluent and 100 mL of Kirk's medium were taken in flasks after adjusting pH at 4.5. The flasks were autoclaved for 15 mints at 121 °C. After cooling to room temperature, the triplicate dye solution containing flasks were inoculated with 5 mL homogeneous spores inocula of respective fungi and incubated at 30 °C for a period of five days. Supernatants obtained after centrifugation (1200 rpm for 10 min.) was run through spectrometer to check absorbance at λ_{max} of 615 nm. The results obtained were recorded the average of three replicates of various intervals.

Optimization of Biodegradation Process

Different environmental and production conditioned effect the microbial production enzyme product and enzyme activities involved in dyestuff biodegradation. Most important of production conditions are pH incubation temperature inoculation size and effect of various amendments [11]. Degradation experiments were run under various production conditions by varying one factor at a time while keeping others factors constant. Different ranges of experimental parameters (pH 3-5; incubation temperature 20-40 °C; inoculum's size 1-5 mL) were selected.

Effect of amendments

After optimization of experimental parameters, effect of different carbon sources like glucose, starch, glycerol, wheat bran, rice bran and nitrogen sources like yeast extract, maize glutein 30%, maize glutein 60%, corn steep liquor, ammonium oxalate was studied to determine their effects on production of fungal enzymes and extent of dyes decolorizing ability of fungus under study.

Decolorization assay via UV-Vis spectroscopy

The decolourization (%) efficiency of the parameters was assessed by using absorbance of UV/Visible spectrophotometer. The following formula was used to calculate decolourization (%) using the following formula

Decolourization % = (I-F) / I x 100
I= initial absorbance
F= Absorbance of decolorized medium

Results and Discussion

Screening of white rot fungi for synthetic dyes effluent

Five different strains of white rot fungi i.e. *S. commune* (SC), *P. ostreatus* (PO), *P. chrysosporium* (PC), *T. versicolor* (TV) and *G. lucidum* (GL) were initially screened. Maximum decolorization was observed on the sixth day of incubation (Fig. 1). The maximum decolorization (72.7%%) was shown by *P. ostreatus* (PO) while *P. chrysosporium* (PC), *S. commune* (SC), *T. versicolor* (TV) and *G. lucidum* (GL) showed decolorization (%), 65.9%, 45.1%, 40.6% and 35.1%, respectively (Fig. 2). Fungal strains involved all ligninolytic enzymes in the decolorization of synthetic textile dyes effluent, however manganese peroxidase (83.9 U/mL) by *P. ostreatus* (PO) was found to be major enzyme produced on sixth day of incubation (Fig. 2). Based on decolorization (%) findings, finally *P. ostreatus* (PO) was selected for advance optimization of several physico-chemical parameters.

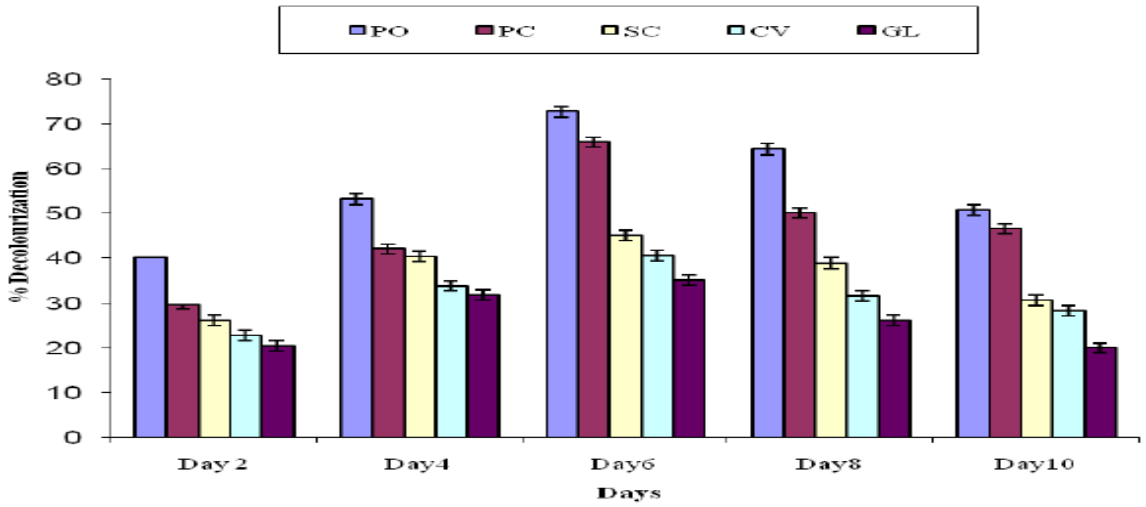


Fig. 2a: Role of white rot fungi in decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

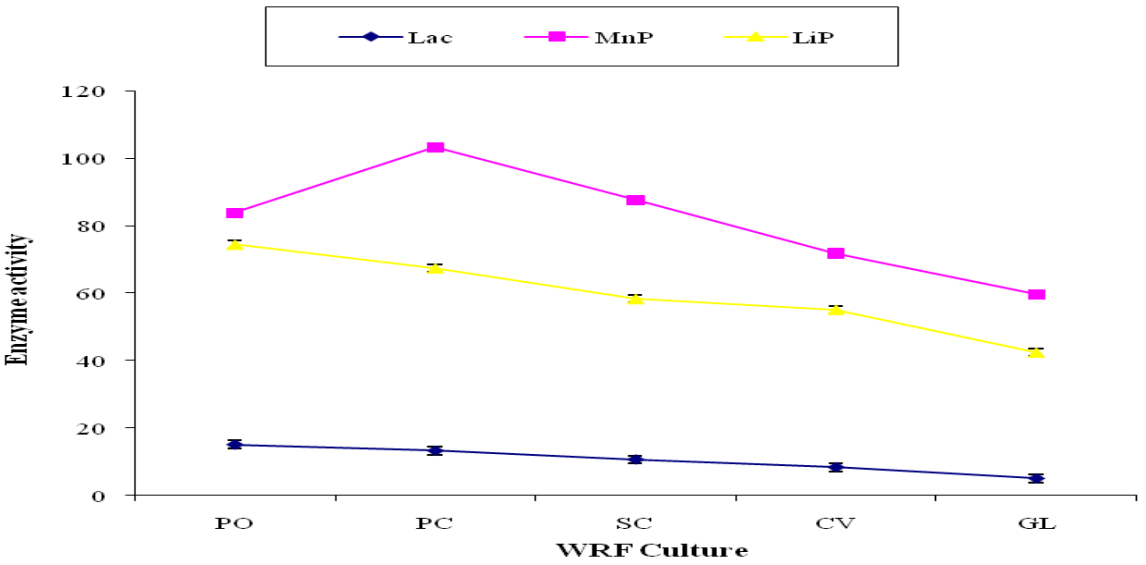


Fig. 2b: Role of ligninolytic enzymes of white rot fungi in decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

These results were supported by work of researches that obtained an induction of the laccase by low $CuSO_4$ concentrations (0.1 and 0.2mM) using *P. ostreatus*, these conditions were not evaluated in this work [12].

Zymes Ligninolytic enzymes of fungi actively degrade the lignin, hence fungal degradation of synthetic dyes proceeds at faster rate [13, 14]. It was reported that mostly laccase is responsible for the enzymatic degradation of textile dyes for by *P. ostreatus* [15]. These enzymes are capable for the degradation of toxins [16], but in present study presence of very fade zones in the decolorization trials suggested that instead of adsorption of dyes, the decolorizatio dye was an enzymatic operation [17]. This conclusion is also agreeable with previous reported work in which it was noticed that the decolorization of colorants was due to breakdown or transformation of specific chromophores by means of *P. ostreatus* [18]. Mycelia synthesis of the fungi generally starts at the first day and decolorization showed up from the 2nd day ahead [19]. Previous studies suggested that dyes degraded by laccases due to cleavage/reduction of azo bond followed by oxidation, de-sulfonation, deamination etc. depending on the nature of dye [20]. Similar results were reported that the presence of RBBR dye induced the production of both the LiP and Lac enzymes by isolate MS8. The increase in ligninolytic enzyme activity, particularly Lac, was most probably needed for the decolourization of RBBR dye by isolate MS8 [21].

Effect of concentration of synthetic dyes Effluent

Dye concentration is an important factor that affects the decolorization process, as high dyes concentration may cause toxic effect that restrict dye decolorization [22]. An enzymatic reaction involves the interaction of the active site of an enzyme with its specific substrate. With an increase in the substrate concentration, the active sites of enzyme molecules get saturated [23]. This result was satisfying to [24] stated that synthetic dyes effluent concentration was increased from 0.01-0.05 (g/100 mL), the percent decolorization was gradually decreased (Fig. 3a and b). While as, increasing the dyes concentration limits the decolorization process to give lower values. This finding is in accordance withresearchers who reported that high dye concentration is a reason of slower decolorization rate [25].

Effect of pH

Effect of pH was examined while keeping other parameters constant. pH was changed from 3.5-5.5. As in highly acidic form, synthetic dyes effluent was decolorized from 40.58 % to 81.11 % (maximum value), when pH was increased from 3.5-4.5. Decolorization (%) was decreased when pH was increased to 5.5. At pH 5.5, the synthetic dyes effluent was decolorized by 47.32 %. The production of MnP by *P. ostreatus* during the decolorization of synthetic dyes effluent was greatest 93.7 (U/mL) at pH 4.5 than other two enzymes (Fig. 4a and b). As observed by researchers, organic acids such as malonate, oxalate yield from the white rot fungi during the early growth age, and later ligninolytic enzymes decomposed them thus, acidic pH is favored for effective decolourization of synthetic dyes wastewater [26]. The dye decolorization is highly dependent on the level of pH which in turn effect the enzymatic activities of certain fungi [27, 28].

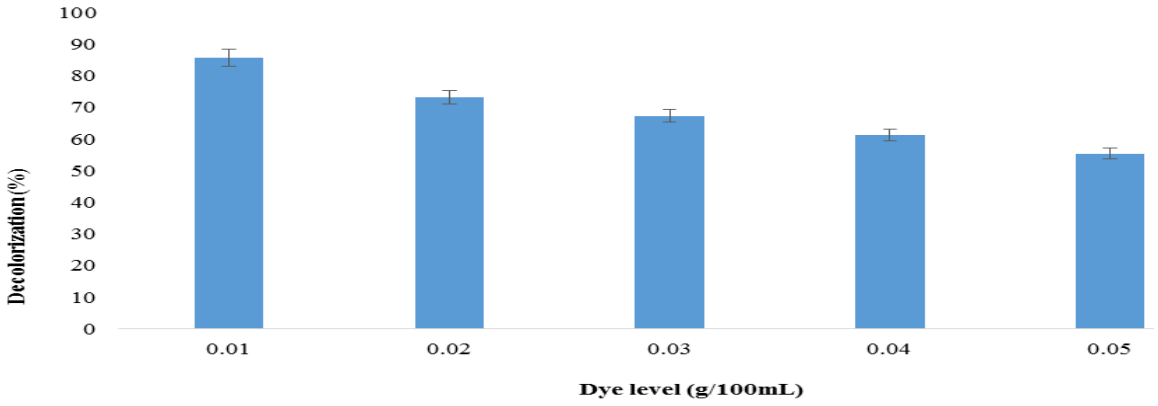


Fig. 3a: Effect of dye level (g/100mL) on decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

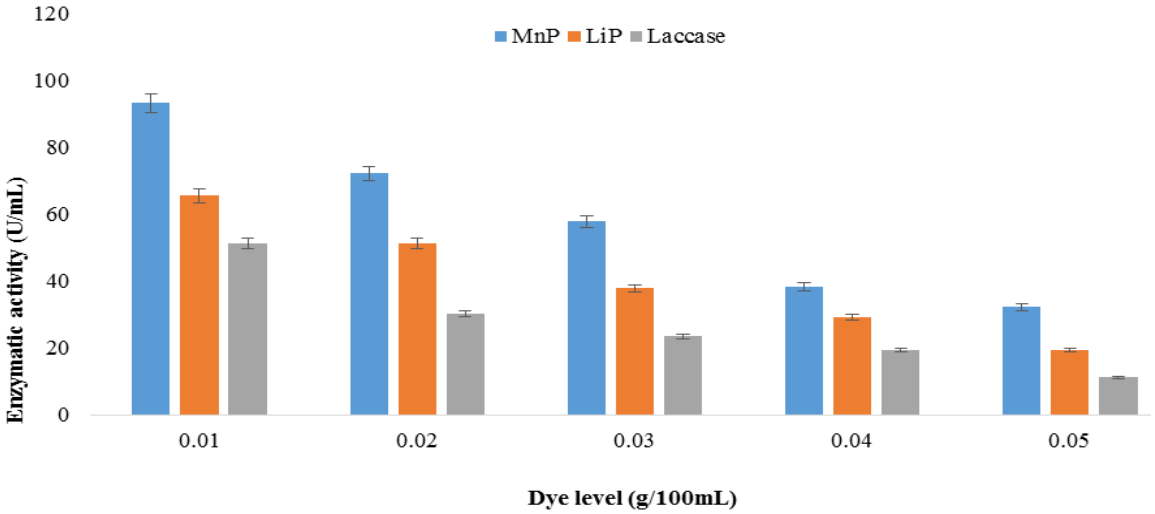


Fig. 3b: Effect of dye level (g/100mL) on activities of ligninolytic enzymes of *P. ostreatus* (b) (λ_{max}= 615 nm).

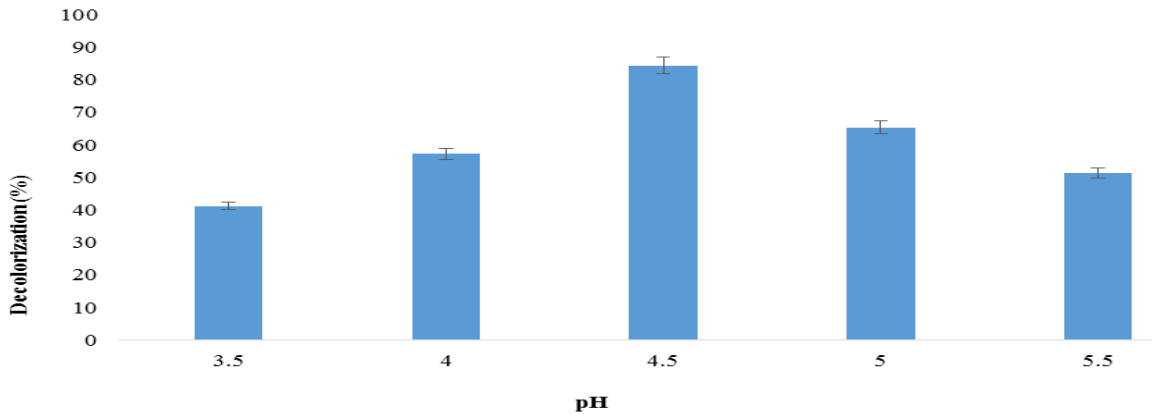


Fig. 4a: Effect of pH on decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

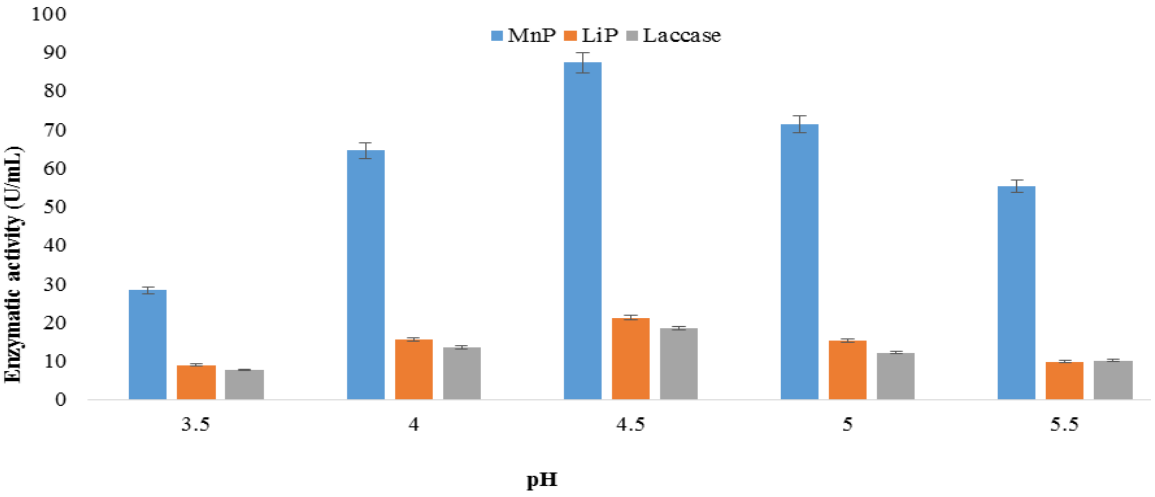


Fig. 4b: Effect of pH on activities of ligninolytic enzymes of *P. ostreatus* (b) (λ_{max}= 615 nm).

Effect of inoculum size

As inoculum size increased, decolourization (%) also increased reaching maximum value 79.36 % at inoculum size 4 mL and further increase in inoculum size brought decrease in its value to 45.16 %. MnP showed maximum production 91.32 (U/mL) while other enzymes like LiP and Laccase showed 15.28 (U/mL) and 14.11 (U/mL) at inoculum size 4 (Fig. 5a and b). In a study it is reported that for the decolorization of the textile effluent inoculum size of 4-5mL is sufficient, beyond this no significant variation in decolorization (%) was recorded. Our results were in consistent with the findings [29, 30].

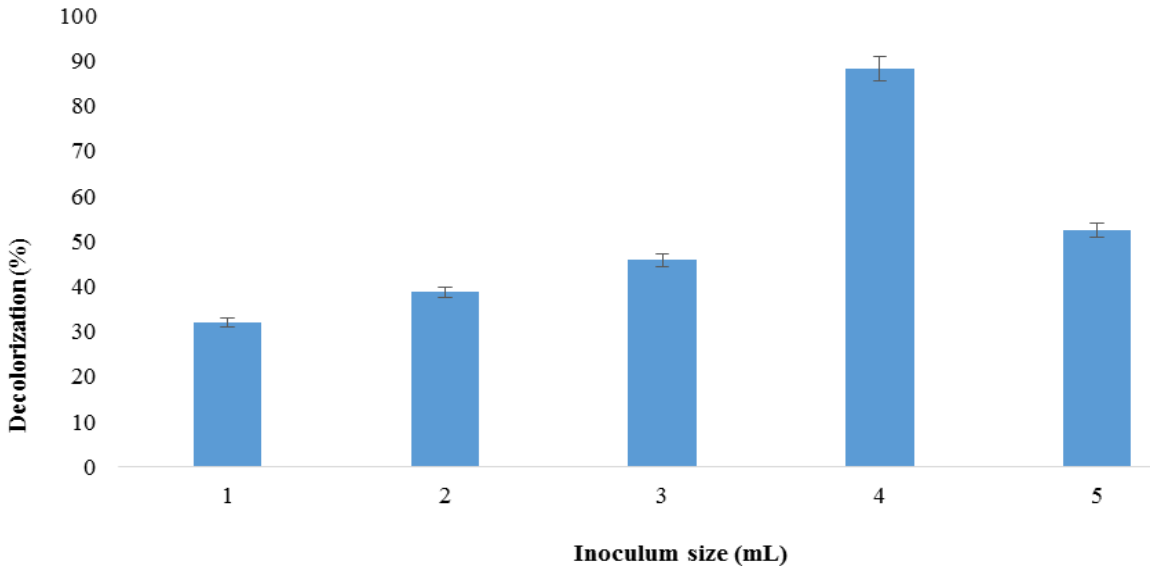


Fig. 5a: Effect of inoculum size (mL) on decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

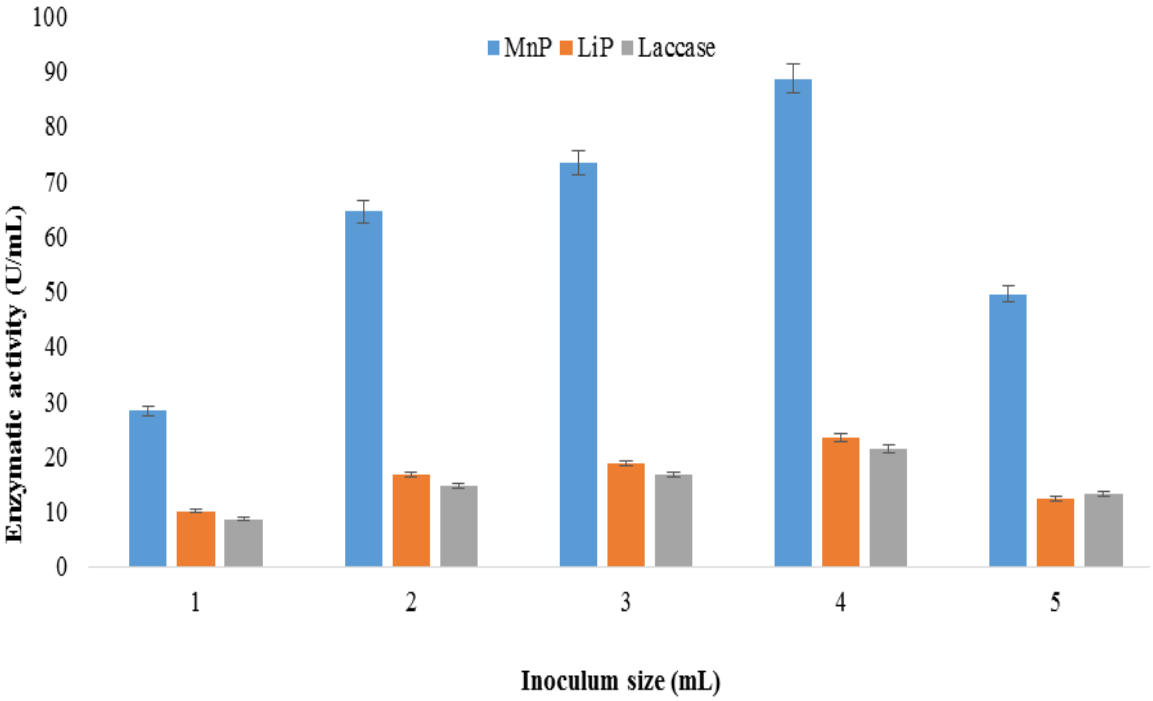


Fig. 5b: Effect of inoculum size (mL) on activities of ligninolytic enzymes of *P. ostreatus* (b) (λ_{max}= 615 nm).

Effect of temperature

Temperature is an important parameter which needs to be optimized as it has a pronounced impact on decolorization of dyes [31]. Other parameters like (synthetic dyes effluent, 0.01%; pH, 4.5; inoculum size, 4 mL) size were kept constant to investigate the effect of varying temperature on production of enzymes for decolourization of synthetic dyes effluent. As temperature increased from 25 to 30 °C, the decolorization of dyes effluent went on increasing, accompanied by highest production of fungal enzymes at this temperature; MnP being the highest in level (Fig. 6a and b). The temperature higher than 30 °C resulted in decrease in decolorization as well as fungal enzymes. 30 °C was found to be optimized temperature exhibiting highest decolorization activity. It was reported that temperature could influenced the mycelial growth of fungi [32-33]. It was related with previous investigation who observed that a laccase produced from a local isolate of *P. ostreatus* had optimum temperature between 30 and 35 °C and rapidly lost activity at temperatures above 40 °C probably due to break down of the structural integrity of laccase protein [34].

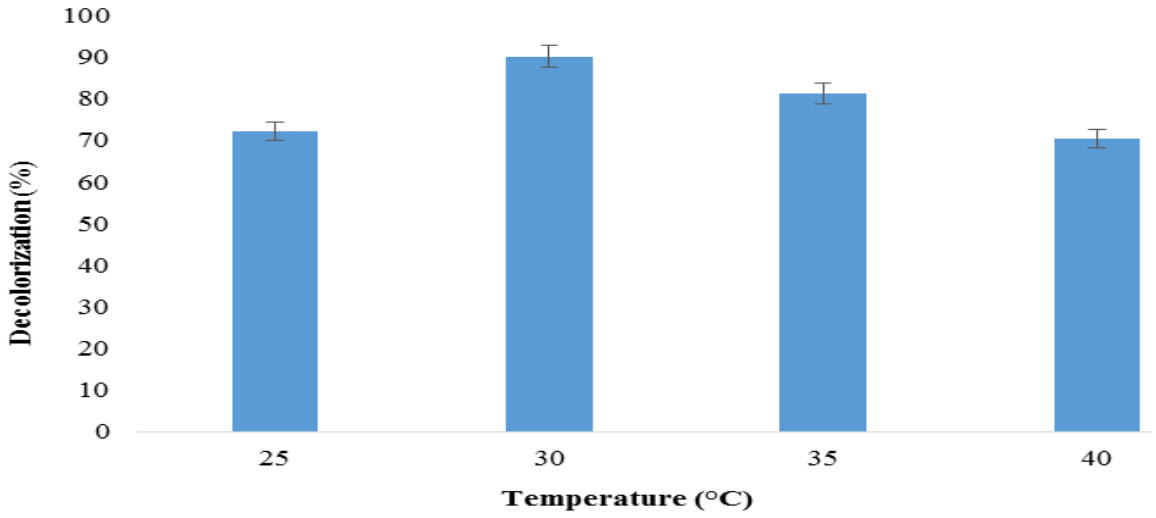


Fig. 6a: Effect of Temperature (°C) on decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

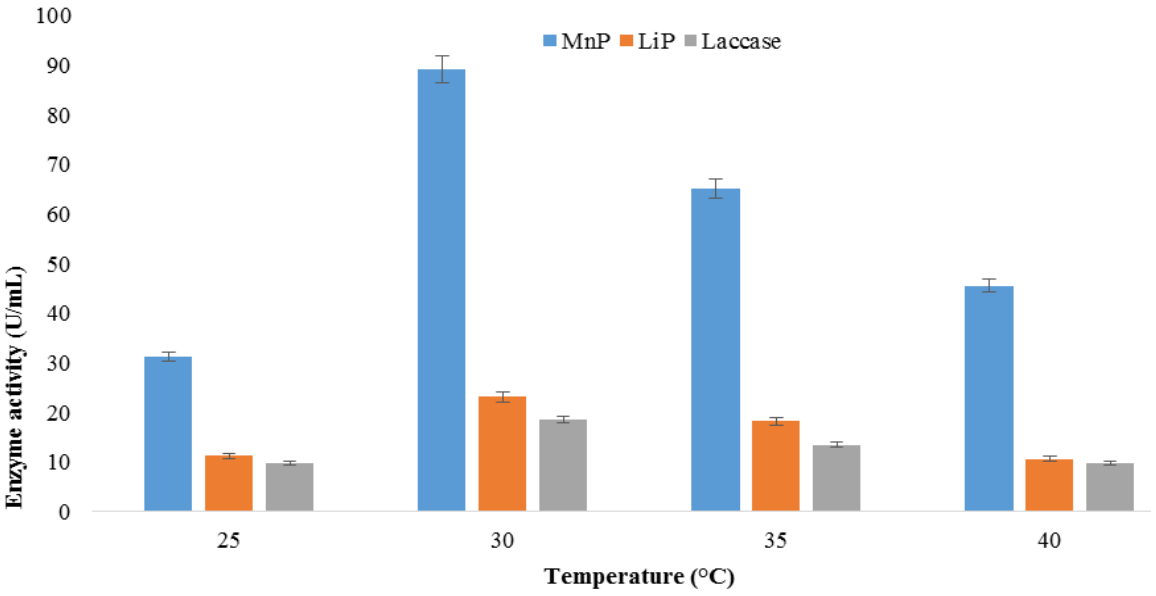


Fig. 6b: Effect of Temperature (°C) on activities of ligninolytic enzymes of *P. ostreatus* (b) (λ_{max}= 615 nm).

Effect of additional carbon sources

Different carbon sources like starch, glycerol, glucose, rice bran and wheat bran etc. were used and maximum decolorization (79.43 %) of synthetic textile dye effluent by *P. ostreatus*was obtained when rice bran was used as carbon source, whereas with starch, glycerol, glucose and wheat bran, the color reductions were 42.23%, 45.94%, 49.01% and 71.19% respectively. In the presence of rice bran as carbon source, the production of MnP by *P. ostreatus* was maximum 95.1 (U/mL). Whereas, maximum production of LiP and Lac was 18.25 (U/mL) and 22.81 (U/mL), respectively (Table-1). Researchers assessed that the basal medium amended with 0, 25, 50, 75 and 100 mM conc. of glucose produced the highest amount of the mycelial growth and maximum decolorization as well [35]. Earlier reports on decolorization studies demonstrated the need of extra carbon sources other than dye itself to upgrade the decolorization procedure. The maximum production of lignin peroxidase and laccase was observed for more effective decolorization [30]. Others revealed that the presence of extra carbon source may results in expanded generation of nucleotides prompting increment in decolorization proficiency [36].

Effect of additional nitrogen sources

Nitrogen is a basic part of microbial growth. Influence of various nitrogen sources like ammonium oxalate, corn steep liquor (CSL), maize glutein meal 60%, maize meal 30% and yeast extract etc. was studied on decolorization of effluent under study (Table-2). Maximum decolorization (72.32 %) of synthetic dye effluent was observed when ammonium oxalate was used as nitrogen source. Urea has been also studied as an economic nitrogen source for dye decolourization by *Ganoderma sp*., *Pycnoporus sanguineus* and *C. tropicalis* cultures [38]. It is evident that less efficient removal of dye with supplemental nitrogen. The supplemental nitrogen being there might cause the decreased microbial activity which diminished the growth and enzymatic activity of fungi, hence decreased the decolorization rate [39].

Effect of mediators

Some dyes under study were not decolorized by laccase enzyme alone, therefore adding redox mediator is important to expand the catalytic activity of laccase towards many recalcitrant compounds [40]. Various redox mediators e.g. veratryl alcohol, $MnSO_4$, glycerol, ethanol, ABTS, oxalate and H_2O_2 were added to evaluate their effect on decolorization of synthetic dyes effluent (Fig. 7a and b). Maximum decolorization (90.12%) was attained when $MnSO_4$ was used as a redox mediator. $MnSO_4$ is an active mediator of MnP (120U/mL) created as a main ligninolytic enzyme by *P. ostreatus* (PO). Researchers reported that 1-Hydroxybenzotriazole is capable to improve the decolorization process of different dyes. Researchers assessed that synthetic dyes are environmental pollutants capable of causing toxicity and carcinogenicity. Employment of laccases either alone or in assistance with mediators were found to be best enzymes for bioremediation of various industrial pollutants [24].

Table-1: Effect of carbon sources on decolorization (%) of synthetic dyes effluent and ligninolytic enzymes of *P. ostreatus* (λ_{max}= 615 nm)

Sources of carbon	% Decolorization ± S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
Starch	42.23 ± 1.48	13.89 ± 1.48	53.34 ± 1.50	14.63 ± 1.47
Glycerol	45.94 ± 1.47	16.67 ± 1.45	65.07± 1.49	16.63 ± 1.46
Glucose	49.01 ± 1.47	17.36 ± 1.48	72.32 ± 1.53	18.92 ± 1.48
Rice bran	79.43 ± 1.46	18.25 ± 1.45	95.1 ± 1.52	22.81 ± 1.43
Wheat bran	71.19 ± 1.45	16.20 ± 1.48	68.24 ± 1.51	21.62 ± 1.42

Table-2: Effect of nitrogen sources on decolorization (%) of synthetic dyes effluent and ligninolytic enzymes of *P. ostreatus* (λ_{max}= 615 nm).

Sources of nitrogen	% Decolorization ± S.E	Laccase (U/mL) ± S.E	MnP (U/mL) ± S.E	LiP (U/mL) ± S.E
Corn steep liquor	51.95 ± 1.55	5.97 ± 1.49	31.93 ± 1.51	10.7 ± 0.44
Yeast extract	57.99 ± 1.53	9.03 ± 1.47	37.1 ± 1.52	11.3 ± 0.42
Maize gluten 30%	62.57 ± 1.53	10.2 ± 1.47	44.61 ± 1.50	12.8 ± 0.45
Maize gluten 60%	70.61 ± 1.52	14.8 ± 1.46	50.49 ± 1.54	14.5 ± 0.43
Ammonium Oxalate	72.32 ± 1.51	15.1 ± 1.46	69.61 ± 1.50	18.4 ± 0.41

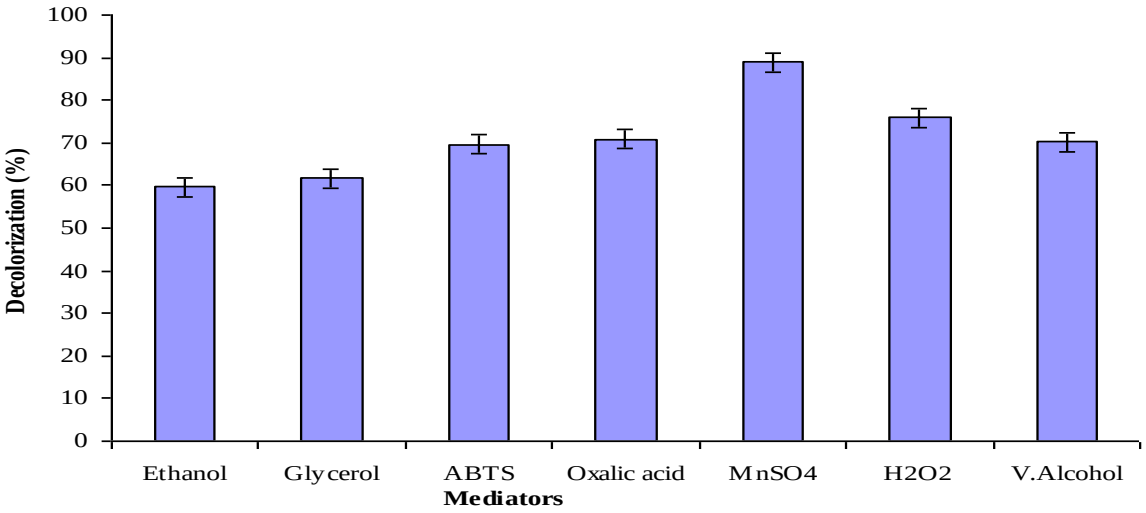


Fig. 7a: Effect of mediators on decolorization (%) of synthetic dyes effluent by *P. ostreatus* (λ_{max}= 615 nm).

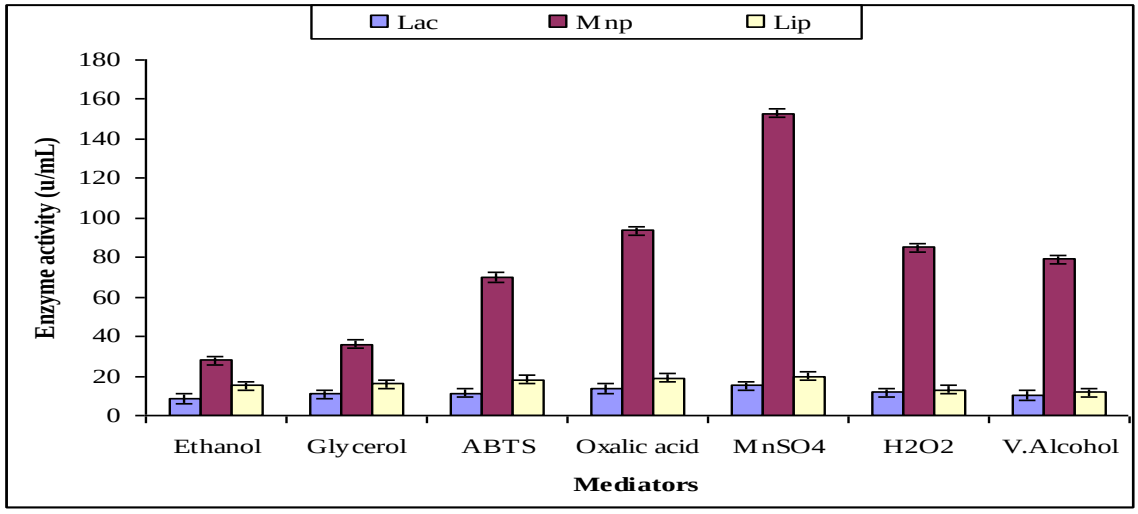


Fig. 7b: Comparison of ligninolytic enzymes produced by *P. ostreatus* during decolorization (%) of synthetic dyes effluent (λ_{max}= 615 nm).

UV-Visible and FTIR analysis of decolorization of synthetic dyes wastewater by P. ostreatus

UV-Visible spectral studies disclosed the decolorization of dyes effluent while FTIR spectral studies helped to reveal degradation of effluent under study. UV-Visible spectral analysis of untreated synthetic dyes effluent demonstrated a peak in the visible region (λ_{max}=615nm) while the treated dyes effluent by PO showed diminution in height of spectral line of absorption maxima. It indicated the decolorization of effluent under study. Moreover, the shift of spectral line in the UV-region confirmed the degradation of effluent (Fig. 8a and b). To characterize the metabolites formed by biological treatment using *P. ostreatus* of dyes effluent, FTIR analysis was done. The FTIR spectrum of untreated dyes effluent exhibited peaks at 3344.59, 2352.21, 1646.3, 619.15 and 613.956 cm^{-1} for –OH stretch of phenol, N=N stretch and C=C stretching of monosubstituted benzene ring (Fig. 9a), however the FTIR spectrum of treated dyes effluent showed peaks at 2364.64 cm^{-1} for =C-H stretch, 1562.06 cm^{-1} for aromatic ring skeleton and 610-690 cm^{-1} for of monosubstituted benzene ring of the FTIR spectrum (Fig. 9b). Lack of peaks between 3650 to 3590 cm^{-1} showed that no formation of phenolic compounds. There were no peaks in the region of 3400 to 3380 cm^{-1} (for –NH stretch) showed that no aliphatic and aromatic amines were formed by *P. ostreatus*

treatment. FTIR spectral analysis specified the degradation of dyes effluent in to intermediate yields. In the decolorized medium, there was no peak in the region of 1646.3 cm^{-1} which is the characteristics of azo bond. Overall results indicated that aromatic amines might be formed in situ which were further undergone to oxidation giving simpler compounds. Our result was supported by literature who stated that HBT-laccase mediator system caused higher decolorization percentage (absorbance reduction) than in case of using laccase alone [41]. These observations are in accordance with the results obtained previously with experiments for optimization of parameters for different dyes decolorization using laccase of *P. ostreatus* ARC280 and HBT- laccase mediator system [27].

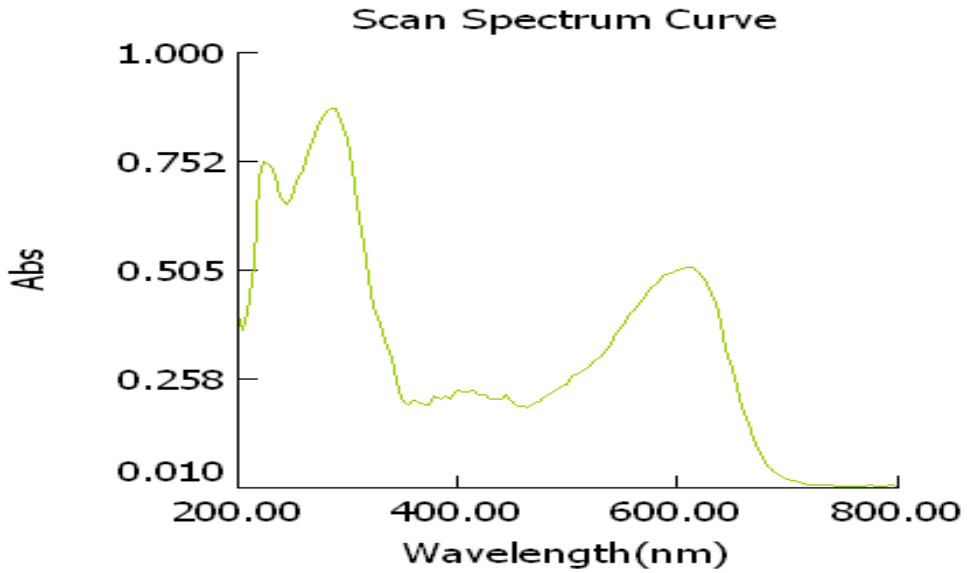


Fig. 8a: UV-Visible spectral analysis of synthetic dyes effluent before biological treatment (λ_{max}= 615 nm).

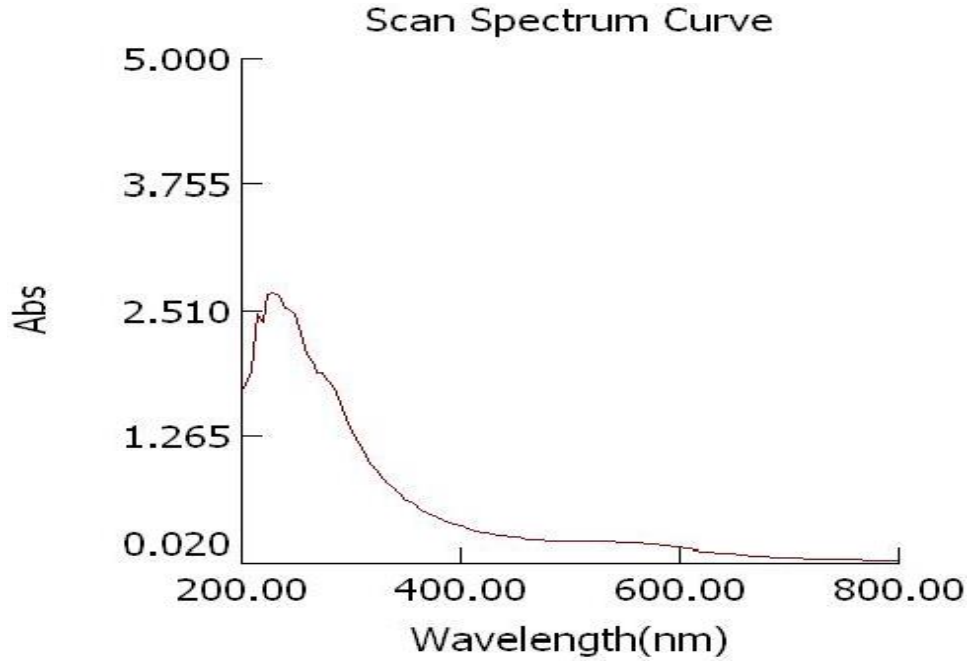


Fig. 8b: UV-Visible spectral analysis of synthetic dyes effluent after biological treatment (λ_{max}= 615 nm).

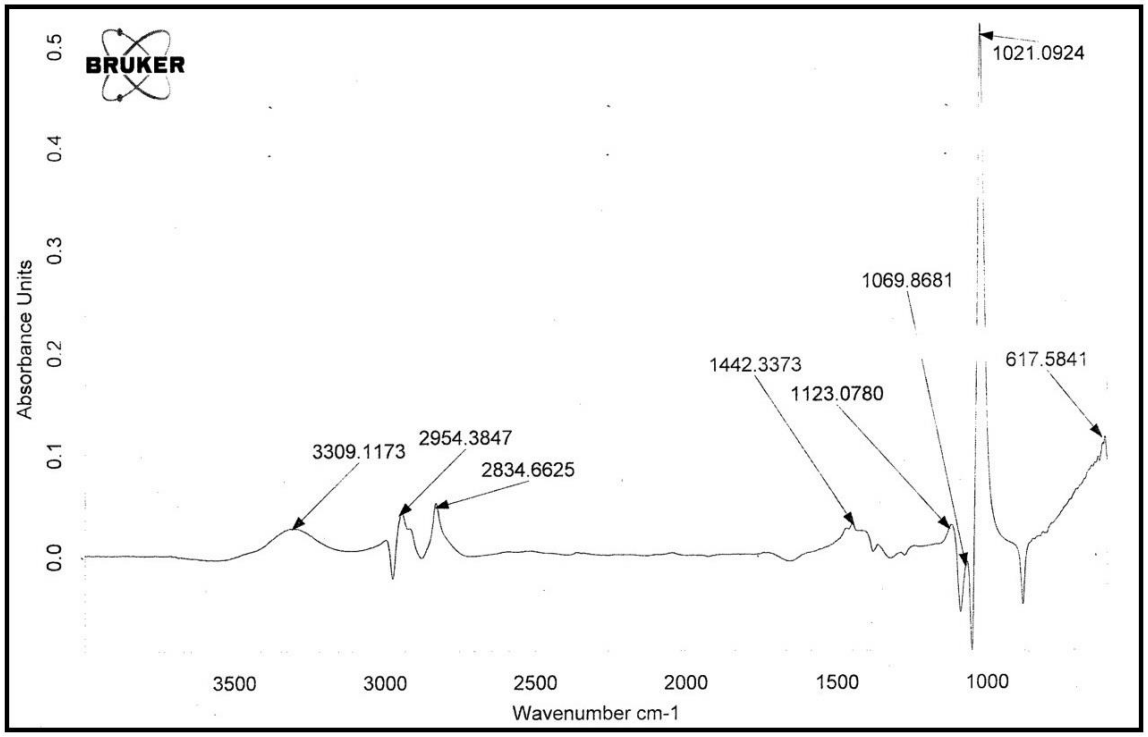


Fig. 9a: FTIR spectral analysis of synthetic dyes effluent before biological treatment (λ_{max}= 615 nm).

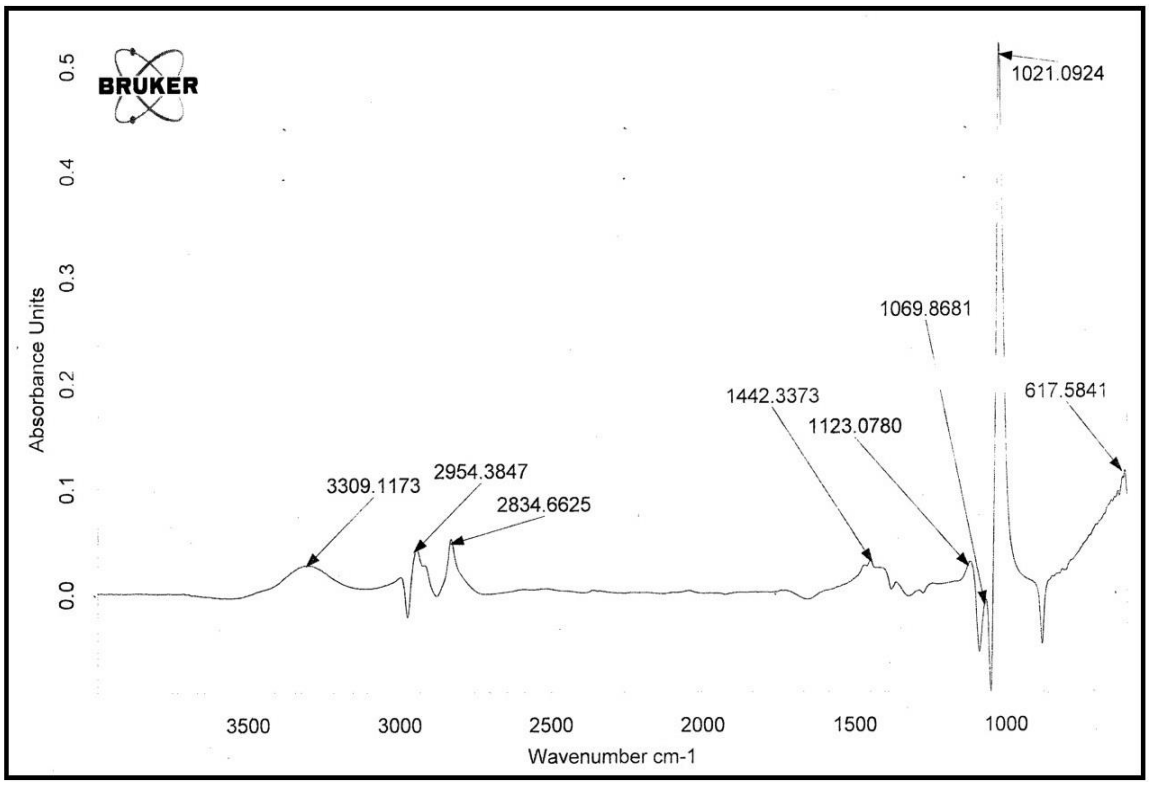


Fig. 9b: FTIR spectral analysis of synthetic dyes effluent after biological treatment (λ_{max}= 615 nm).

Conclusion

Biodegradation as compared to physical or chemical degradation of synthetic dyes effluent was found to be more efficient technology in terms of its eco-friendly nature and complete degradation of almost all notorious dyes. *Pleurotus ostreatus* reasonably exhibited better decolorization capability for synthetic dyes effluent. Ligninolytic enzymes are the key factors responsible for the degradation of azo dyes which work well under optimized growth conditions. The results disclosed that *P. ostreatus* can be effectively used for efficient degradation of azo dyes.

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