Final Report

UGC Project

# Bioremediation of synthetic dyes pollution by white rot fungi (*Pleurotus* spp.)

UGC Reference No. (F. No. 41-1126/2012 (SR)

Dated: 25-07-2012

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**ABSTRACT**

In the present investigation the efficiency of four species of white rot fungus - *Pleurotus* (*P. flabellatus, P. ostreatus, P. sajor-caju* and *P. citrinopileatus*) and their improved dikaryons (heterokaryons) were studied for decolorization of five azo dyes (Acid black, Congo red, Methyl orange, Methyl red and Phenol Red) and production of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase enzymes. These species of *Pleurotus* were selected out of nine species. Among the five dyes, Methyl orange decolourization was faster and earlier i.e. 96.98% on the 20$^{th}$ day on incubation by *P. citrinopileatus,* whereas remaining four dyes took 25 days for decolourization. 97.42% Congo red, 90.67% Methyl red and 84.52% Acid black were decolourized by *P. flabellatus*, and 93.33% Phenol red was decolourized by *P. citrinopileatus* on 25$^{th}$ day of incubation. Improved dikaryons decolourized the dyes more effectively.

**INTRODUCTION**

A vast amount of dyes are produce annually with a variety of color and chemical structure around the world. These synthetic organic compounds with multiple aromatic rings are either fused or connected covalently and modified with various hydrophilic functional groups such as amine, carboxylic acid, carbonyl and hydroxyl group to produce desired color and increase affinity to the material being dyed. These dyes are generally employed as coloring agent in food, pharmaceutical, woolen, paper, metal, cosmetic and textile industries (1). The textile industries are major consumer of these dyes and account for two-thirds of the total dyestuff market. The increasing use of synthetic dyes is alarming and their discharge as textile waste may cause substantial ecological damage. Many synthetic dyes are nontoxic, some of their degradation by products such as anilines are often more toxic and sometimes carcinogenic than the parent compounds. In addition to the toxicity, residual organic dye can also exert various hazards on the aquatic ecosystem by depleting dissolved oxygen, blocking sunlight penetration, inhibiting photosynthesis and growth of aquatic organism (2,3).

Removal of color from dye containing wastewater is a current issue of discussion and regulation in many countries, because water is a viable asset that should be protected (4). The conventional physical and chemical methods possess inherent limitations such as high cost, formation of hazardous byproducts and intensive energy requirement (5,6). In spite of the existing physical/chemical technologies, biological process provide an alternative, because they are cost effective, eco-friendly and can be applied to wide range of dye containing effluents (7,8,9). Currently, a lot of studies have focused on white rot fungi (WRF) than seem

to be more prospective organism (10,11). Due to extracellular nonspecific enzyme system WRF are so far exclusive in their strong oxidative capability. *Pleurotus* is a WRF belonging to the family *Pleurotaceae* produce two major families of enzymes generally termed ligninolytic enzymes i.e. extracellular peroxidase (Manganese Peroxidase, MnP; Manganese Independent Peroxidase MIP; Lignin Peroxidase, LiP and Versatile Peroxidase, VP) and Phenol oxidase (Laccase). However, not much attention has been raised for its decolorization ability. The current work is to source out the capability, efficacy and efficiency of the *Pleurotus* species and its improved dikaryons (heterokaryons) in production of LiP, MnP and Laccase enzymes and in decolorizing the synthetic textile dye such as PSP on different pH, age and concentration of inoculum, surfactants viz. sodium dodecyl sulphate and Tween-80. This research is also indirectly contributing knowledge to wastewater treatment in textile industries.

## MATERIALS AND METHODS

### *Cultures and their maintenance*

The pure cultures of *P. flabellatus, P. ostreatus, P. sajor-caju* and *P. citrinopileatus* used in present experiments were procured from Directorate of Mushroom Research, Solan and Indian Agricultural Research Institute, New Delhi. Throughout the study, the stock cultures were maintained on potato dextrose agar (PDA) slants at $25^0$C and sub-cultured at regular interval of three weeks.

### *Production of enzymes*

The experiment on production of ligninolytic enzymes was carried out in potato dextrose broth medium (20% peeled potato and 2% dextrose). Double distilled water was used for preparation of the medium and pH was adjusted at 6.0 by using N/10 NaOH or N/10 HCl. Incubation was carried out at $25^0$C in BOD incubator in cotton plugged 250 ml Erlenmeyer flask containing 100 ml of media. Each flask inoculated with 1 mm in diameter of agar pieces of *Pleurotus* species and improved dikaryons from actively growing area on potato dextrose agar plate.

### *Extraction of extracellular enzymes*

Samples of substrate were collected at regular interval of 5 days and extracted in phosphate buffer (pH 6.0) for ligninolytic enzymes. Filtrate of extraction was used for enzyme assay.

### *Enzymatic study*

### *Lignin Peroxidase (1.11.1.14)*

Lignin peroxidase activity was determined using veratryl alcohol as substrate. The reaction mixture contained 1 ml of crude enzyme extract, 0.5 ml of 2 mM veratryl alcohol,

1.5 ml of 0.1 mM Sodium tartrate buffer (pH 2.5) and 0.2 ml of 0.4 mM $H_2O_2$. The oxidation of substrate was followed by spectrophotometrically at $\lambda_{max}$ 310nm. One activity unit was defined as 1µmol of veratryl alcohol oxidized per minute.

***Manganese Peroxidases (EC 1.11.1.13)***

Manganese peroxidase (MnP) activity was determined using guaiacol as substrate. The reaction mixture contained 0.2 ml of 0.5 M Na-tartrate buffer (pH 5.0), 0.1 ml of 1 mM $MnSO_4$, 0.1ml of 1mM $H_2O_2$, 0.25 ml of 1 mM guaiacol and 0.3 ml of crude enzymes. The oxidation of substrate at $30^0$C was followed spectrophotometrically at $\lambda_{max}$ 465nm (12).

***Laccase (EC 1.10.3.2)***

Laccase activity was determined via the oxidation of o-methoxyphenol catechol monomethylether (guaiacol) as substrate. The reaction mixture contained 1 ml of 1mM guaiacol in 0.1M sodium phosphate buffer (pH 6.0) and 1ml of crude enzyme solution was incubated at $30^0$C for 10 min. The oxidation was followed by the increase in absorbance at $\lambda_{max}$ 495nm. (13).

**Fructification and basidiospore isolation**

***Cultivation***

The method of spawn, substrate preparation and spawning were described in our earlier published paper (14, 15) and illustrated in Figure 1.A-C.

***Spore Print***

The dropping spores were selected from healthy and young fruit bodies to prepare spore prints. The cap of the mushroom fruit body was cut down and kept on sterilized paper, on the sterilized petriplate, with gills down (Figure 1.D). The petriplate was then sealed properly with cello tape and the entire setup was placed in an undisturbed area for overnight. When the cap was removed, the spore prints were collected in the petriplate on paper. Then the resulted spore prints of *Pleurotus* species were stored at $4^0$C for their use in single spore isolation.

***Germination and isolation of homokaryons***

Paper bearing spores was cut into 2x2 cm size and suspended in 0.5% NaCl in 100 ml sterilized double distilled water and agitated at 150 rpm in orbitary shaker for 2 hour to make uniform suspension (Figure 1.E). The spore suspension further serially diluted up to $10^{-4}$ dilution from which 150 ml of the spore suspension was transferred and spread to each petriplate containing 18-20 ml of solid agar medium under aseptic condition. The inoculated petriplate were incubated at $25^0$C in BOD incubator for one week. After germination of single spore (Figure 1.F) marked with the help of permanent marker on backside of petriplate, it was lifted with the help of a fine tip of inoculation needle and transferred to another petriplate containing 18-20 ml potato dextrose agar medium under aseptic condition. The

single spore colonies were confirmed by lacking of clamp connection through microscopy. Then these colonies were sub-cultured on PDA slants and incubated at $25^0$C in BOD incubator for further use.

***Hyphal anastomosis***

At least 12 monokaryons from four selected *Pleurotus* species were randomly selected and 144 combinations of the above cultures were stabilised for compatibility test. The mating compatibility between homokaryotic cultures were performed in duel culture technique by placing actively growing mycelia (1mm in diameter) of single spore cultures of above two strains approximately 1cm apart in the center of a 90 mm petridish of potato dextrose agar . Three replicates were used for each combination and arranged in a completely randomized design. A total of these 16 groups will be obtained from four selected species. After that these 16 groups will be mated with each other to develop heterokaryons. In each step crosses will be confirmed through clamp connection under 100X magnification with suitable stain. After the confirmation a sample of mycelia was transferred to fresh agar medium for further examination of dye tolerance level in liquid broth.

***Decolorization studies in liquid media***

The mycodecolorization experiments were done in potato dextrose broth medium supplemented with PSP 300 mg/l. Each inoculated with screened species and improved strain of *Pleurotus* in 250 ml Erlenmeyer flask containing 100 ml media and incubated in stagnant condition in BOD incubator at $25^0$C. Dye disappearance was detected spectrophotometrically (Elico 164-SL) at $\lambda_{max}$ 497 nm for PSP after $20^{th}$ days of incubation. Results were reported as the mean value of percent dye decolorization (% DD) for three replicates (16).

***Optimization of parameter for PSP decolorization***

**Effect of pH**

All *Pleurotus* species and heterokaryons (dikaryons) were incubated with dye containing liquid broth medium to evaluate maximum dye decolorization at different pH value ranging from 5.0, 5.5 and 6.0. The pH was determined with electronic pH meter model- 361. Before sterilization of media, their pH was adjusted to the required level using N/10 NaOH or N/10 HCl.

**Effect of age and concentration of inoculums**

Mycelial bits of 1 mm in diameter were inoculated in 100 ml of potato dextrose broth medium in 250 ml Erlenmeyer flasks and incubated in BOD incubator at $25^0$C for 10 and 15 days. After the maximum growth of mycelia, homogenize suspension was made at 150 rpm in orbitary shaker with the help of sterilized small glass pieces. The mycelial suspension was

then inoculated in 100 ml Erlenmeyer flask, containing 30 mi dye containing broth medium at the concentration of 3 mi and 5 mi and incubated in BOD incubator for observing the dye decolorization (17).

**Effect of surfactants**

Two types of surfactants - anionic surfactant i.e. Sodium dodecyl sulphate (SDS) and nonionic surfactant i.e. Tween-80 were used with the dye. The concentration varied from 0.5 mM, and 1.0 mM of SDS, 0.1% and 0.2% of Tween-80 in 100 ml Erlenmayer flasks containing 30 ml dye in broth medium. Mycelial bits of 1 mm in diameter of *Pleurotus* species and improved dikaryons was inoculated and incubated in BOD incubator at $25^0$C for observing the dye decolorization (18).

**Effect of Aromatic Amino Acid:**

The effects of aromatic amino acids on dye decolourisation were tested. The stock solution of Tyrosine, Tryptophan and Phenylalanine were sterilized by membrane filter and stored in dark brown amber bottles. These amino acids were added to pre-sterilized 100ml erlenmayer flask, containing 30ml dye potato dextrose broth medium at various concentration viz. 0.2 µM and 0.4 µM. The inoculums of *Pleurotus* strains was inoculated and incubated at 27± $2^0$ C for observing dye decolourization.

**Effect of Trace Element**

To study the effect of trace elements like $CuSO_4.5H_2O$, $MnSO_4.H_2O$ and $ZnSO_4.7H_2O$ at the concentration of 25ppm and 50ppm were selected for dye decolourization. These trace elements were added at above concentration in 100ml erlenmayer flask containing 30ml dye Potato Dextrose broth medium and selected *Pleurotus* strains were inoculated and incubated in BOD incubator at 27± $2^0$ C for observing dye decolourization.

**Results**

**Screening and Selection of *Pleurotus species*:**

All the nine species of *Pleurotus* showed slow mycelial growth in the initial stage i.e., on 3rd day and fast growth after 3rd day. They took 12 days to fill the petridish and showed different rate of decolourization except *P. citrinopileatus*, which took 9 days to fill the petri dish and caused maximum decolourization. *P. flabellatus*, *P. ostreatus* and *P. sajor-caju*, took 12 days to cover the whole petridish. Whereas, *P. florida*, *P. sapidus, P.roseus* and *P. eryngii* showed relatively slow decolourization rate and took 15 days. The vegetative growth of *P. fossulatus* was inhibited and mycelia took 18 days to complete decolourization.

**Enzymatic study**

The activity of lignin peroxidase, Manganese activity and Laccase were determined under *in vitro* condition on Acid black, Congo red, Methyl orange, Methyl red and Phenol red. As

mentioned in the materials and methods earlier, test samples for enzyme assay were taken from liquid culture containing 0.02% dye inoculated with selected *Pleurotus* species. Enzymatic analysis was carried out at 5 day interval. During this study four most promising species of *Pleurotus* were identified as better decolourizer of Poly R-478 dye among the nine species on solid medium. The selected four species (*P. flabellatus*, *P. ostreatus*, *P. sajor-caju*, and *P. citrinopileatus*) were carried out for the five azo dye (Acid black, Congo red, Methyl orange, Methyl red and Phenol red) decolourization and activities of ligninolytic (LiP, MnP and laccase) enzymes. The higher activity of LiP was observed by *P. citrinopileatus* in liquid media supplemented with Acid black followed by *P. flabellatus* on Congo red, Phenol red and Methyl orange, while the lowest activity was recorded on Methyl red by *P. citrinopileatus. P. citrinopileatus* showed maximum MnP activity in liquid media containing Methyl orange and Acid black followed by *P. flabellatus* on Phenol red and Congo red, whereas the miniumum MnP activity was recorded on Congo red by *P. flabellatus.*

In Methyl orange supplemented liquid media, *P. ostreatus* showed maximum laccase activity followed by *P. citrinopileatus* on Acid black, Phenol red and Methyl red and the minimum laccase activity in Congo red containing media. Among the five dyes, Methyl orange decolourization was faster and earlier i.e. 96.98% on the 20$^{th}$ day on incubation by *P. citrinopileatus,* whereas remaining four dyes took 25 days for decolourization. 93.33% Phenol red decolourized by *P. citrinopileatus* on 25$^{th}$ day of incubation.

***Ligninolytic enzymes***

The lignin peroxidase activity of all the five species including basidiospore derived dikaryons is given in Fig 2. After 5 days of incubation Poc 9X6 showed 28.55 U LiP activity whereas, *P. ostreatus*, *P. citrinopileatus, P. flabellatus* and Pfo 6X9 showed 18.97U, 19.54U, 20.23U and 22.10U, respectively. After 15 days, improved dikaryons Poc 9x6 showed maximum LiP activity i.e., 72.80U followed by *P. flabellatus, P. citrinopileatus,* Pfo 6X9 and *P. ostreatus.*

Fig. 3 shows manganese peroxidase activity of *Pleurotus* species and their basidiospore derived dikaryons. After 5 days of incubation Poc9X6 showed 315.70U MnP activity whereas, *P. ostreatus, P. flabellatus, P. citrinopileatus* and Pfo 6X9 showed 198.54U, 270.33U, 289.37U and 290.80U, respectively. During time course of culturing, basidiospore derived dikaryons Poc 9X6 showed maximum LiP activity i.e., 615.15U on 10$^{th}$ day of incubation, followed by *P. flabellatus, P. ostreatus, P. citrinopileatus* and Pfo 6X9.

Laccase activity of all the *Pleurotus* species and basidiospore derived dikaryons is presented in Fig 4. Among all five species including dikaryons, after 5 days Pfo 6X9 achieved 425.92U laccase activity, whereas *P. ostreatus, P. citrinopileatus, P. flabellatus* and

Poc 9X6 showed 297.33U, 347.78U, 358U and 420.15U, respectively. After 10$^{th}$ day, the improved dikaryons Poc 9X6 showed maximum laccase activity i.e., 715.25U followed by Pfo 6X9, *P. citrinopileatus, P. flabellatus* and *P. ostreatus.*

***Optimization of parameter for dye decolorization***

***Effect of pH***

Different pH was studied for enzymes activity and decolorization of PSP by *Pleurotus* species and improved dikaryons at pH 5.0, 5.5 and 6.0 is shown in fig. 5. Among the all three pH, best result in term of decolorization was achieved at pH 5.5 by Poc 9X6 followed by others.

***Effect of Age and concentration of inoculum***

Fig 6 illustrates the effect of mycelial age and concentration of all *Pleurotus* species and improved dikaryons for PSP decolorization. The maximum decolorization was gained by 5 ml 10 days old culture of Poc 9X6 followed by others.

***Surfactant***

The effect of surfactants i.e., SDS and Tween-80 on *Pleurotus* species and improved dikaryons for PSP decolorization is depicted in Fig 7. 0.1% Tween-80 and 0.5 mM SDS supported to Poc 9X6 for maximum decolorization in comparison to others.

**Strain Improvement:**

The strain improvement program was started with the isolation of 12 monokaryones from each species of *Pleurotus* by the spore print generation. 48 monokaryons (homokaryons) were tested for mating response, further the monokaryons with similar mating behavior were sorted out and were rearranged and classified into four distinct groups (A1B1, A1B2, A2B1 and A2B2). A total of 16 groups were obtained from four selected species and these groups mated with each other to develop dikaryons (heterokaryons). Then these developed dikaryons were screened on agar plate containing 0.02% Poly R-478. Four most promising dikaryons (heterokaryons) i.e. Pfo 6X9. Poc9X6 and Pscc 8X3 were selected on the basis of higher growth rate (radial growth) and decolorization on potato dextrose agar media containing screening dye.

After the selection of most promising dikaryons (heterokaryons), these were inoculated in liquid medium supplemented with Poly R-478 at the concentration of 0.02%, 0.025% and 0.03% for the evaluation of tolerance level. At the higher concentration Poc 9X6 and Pfo 6X9 showed better decolorization, whereas less or no decolorization was found by Pscc 8X3 and Posc 4X6.

**Effect of Amino acids on enzymes activities and Acid black decolourization:**

The increased LiP activity if improved dikaryon (heterokaryon) was observed in the Acid black containing media supplemented with the 2.0 µM Tryotophan, whereas 2.0 µM tyrosine assisted the maximum MnP and laccase activity by improved heterokaryons in the Methyl orange and Acid black containing media, respectively. The maximum Phenol red decolorization was also found by improved dikaryon in the presence of 2.0 um Tyrosine.

**Effect of Trace elements on enzymes activities with Acid black decolourization:**

The effect of trace elements like Copper (Cu), Manganese(Mn) and Zinc (Zn) at 25ppm and 50ppm on the improved dikaryons of *Pleurotus* species for Lignin peroxidase, Manganese peroxide, Laccase and decolorization of Acid black were analyzed at regular interval of 5 days over the incubation period of 25 days. It was found that the lower concentration of Zn supported the maximum LiP activity, while the MnP activity was higher in the presence of Mn at lower concemtration. The laccase activity and decolourization of the dye was achieved by improved heterokaryons in the presence of Cu at lower concentration.

The main finding of the present study is that the improved dikaryons (heterokaryons) showed better ligninolytic enzymes activities and decolourization of the above mentioned dyes in comparison to parent (*Pleurotus*) species. It was also found that all studied parameters i.e. pH, age and concentration of inoculum, surfactants, aromatic amino acids and trace elements, markedly affect the enzymes activity and dye decolourization.

**Discussion**

In the present study four most promising species of *Pleurotus* i.e. *P. flabellatus*, *P. ostreatus*, *P. sajor-caju* and *P. citrinopileeatus* were selected out of nine, which showed complete decolourization of Poly R-478 on the $12^{th}$ day of incubation. Out of four dikaryons investigated for dye decolourization, two heterokaryons Poc 9X6 and Pfo 6X9 performed better than the other two dikaryons i.e. Pfsc 8X7 and Pfc 4X6. Heterokaryons Pfo 6X9 and Poc 9X6 decolourized the dye completely in 9 days, whereas Pfsc 8X7and pfc 4X6 decolourized it in 12 days. The probable reason for the fast decolourization of Poly R-478 by improved dikaryons than parental (*Pleurotus*) species may be due to increased biomass production led to higher extracellular enzymatic activity. The decolorization of dyes mainly depends upon the enzymes production by microbes. Faster dye degradation by the white rot fungus *P.chrysosporium* has been observed by other workers as well. Several known white rot fungal species i.e. *I. lacteus*, *P. ostreatus*, *T. versicolor*, *P. chysosporium* and *Bjerkandera* species are capable of efficient decolorization of a broad spectrum of chemically different dyes.

The lignin modifying enzymes (LMEs) i.e. lignin peroxidases (Lip E.C. 1.11.1.14); magnese peroxidases (MnP.E.C.1.11.1.13) and laccases (Lac, E.C.1.10.3.2), are produced by

some WRF while, other produces only one or two of them (18, 19). There are two major families of ligninolytic enzymes which are involved in lignolysis: peroxidases and laccases (20-22). These enzymes are capable of forming radicals inside the lignin polymer, which results in destabilization of bonds and finally in the breakdown of the macromolecule of lignin (23). *Pleurotus* species have been reported the produce all the three modifying enzymes, which play a vital role in biodegradation and bioremediation (14, 15, 24). Ligninolytic enzymes are produced in the initial stage while, cellulolytic and xylanolytic enzymes are produced in the later stage of growth of *Pleurotus* species (24, 25). The improved dikaryons (heterokaryons) exhibited maximum LiP activity than all the *Pleurotus* species. Eichlerova et al. also reported similar observations that the isolates showed higher production of lignolytic enzyme in comparison to parental strain in synthetic dyes containing medium (26). The observation of tolerance level of Poly R-478 by improved dikaryons (heterokaryons) (Table 4.28), indicate that the Pfo 6X9 and Poc 9X6 decolourized the dye at the concentration of 0.02%, 0.025% and 0.03%. The poor decolourization was observed by Pocc 8X3, whereas Posc 4X6 at 0.03% failed to decolourized Poly R-478. Dye concentration had a negative effect on decolourization rate in general. Due to toxicity of the dyes to the microbial cells, the production of microbial biomass remains lower at higher dye concentrations. LiP catalyzes several oxidations in the side chains of lignin and related compounds by one-electron abstraction to form reactive radicals (27, 28). Also the cleavage of aromatic ring structures has been reported (29). LiP are not essential for the attack on lignin: several highly active WRF and litter-decaying fungi (e.g. *Ceriopsis subvermispora, Dichotomitus squalens, Panus tigrinus, Rigidosporus lignosus*) do not excrete this enzyme (30-33). The higher MnP activity was observed by improved dikaryons whereas, the parental (*Pleurotus*) species showed less MnP activity in PSP containing media. The basidospore derived monokaryotic isolates is an efficient method of reaching higher variation in the production of MnP (34,35). The principle role Mn2+ to Mn3+, which then binds to appropriate ligands, diffuses from the enzyme and in turn oxidizes phenolic substrate (36-38). Lignin as well as recalcitrant xenobiotics such as nitroaminotoluenes (23,39) and synthetic textile dyes (17). The improved dikaryons (heterokaryons) showed higher laccase activity in PSP containing medium than the parental species. Laccase and other lignolytic enzyme showed higher production on dikaryons of *Pleurotus* species obtained after crossing of compatible basidiospore-derived monokaryons selected from the parental basidiospore population on the basis of exceptionality in enzyme production (34,40). Decolourization of synazol red (azo red) by *P. ostreatus* is 96% in 24 days at pH 5.5 whereas, the enzyme exhibited highest activity at pH 6.0 (15). Srivastava et al., reported pH 5.5 as best for

decolourization of Direct red by improved strains of *Pleurotus* species (41). Dominguez et al. (6) reported that, pH 4.5 supported higher peroxidase activity by *P. chrysosporium* on media containing Poly R-478. 8 ml 4 days old mycelia suspension of white rot fungi was effective for decolorization of Acid yellow 99, Acid blue 300, and Acid red 114 (17). Maximum decolorization of Direct red was achieved by 5 ml of 10 days old culture of *Pleurotus* and its improved strains. According to Mittar et al, the maximum decolorization of paper and pulp mill effluents could be seen by using 20% of 7 days old culture of *P. chrysosporium* (42). Urek, et al reported, 0.05% of Tween-80 supported highest MnP activity (37). Tween 80 contains an oleat (an unsaturated fatty acid) which can peroxidised by MnP and the oxidant so generate could participate in organo- pollutant degradation by fungal culture (43). According to Srivastava et al., 0.1% Tween-80 supported maximum decolorization of Direct red (19).

In the present investigation, the effect of trace element on the improved dikaryons (heterokaryons) for dye decolorization was studied. 25 ppm of Cu supported maximum decolorization of acid Black 0.03% than Mn and Zn. Because copper is a prosthetic group of laccase enzyme and the causative agent of reactive oxygen species (ROS) which enhances the degradation of the dye. The minimum decolorization of Congo red by improved dikaryon was observed in the presence of 25 ppm of Zn. The significant increase in and decolorization efficacy at 10 dikaryon copper is the well-known fact that copper is an inducer of laccase. The expression of laccase in the white rot fungus is generally regulated at the level of gene transcription by copper. Lignolytic enzymes and ROS have reacted synergistically and improved the dye decolorization.

**Summary**

The summary of the present investigation on selection and improvement of the *Pleurotus* species for azo dye decolorization along with the enzyme activities are given below.

During this study four most promising species of *Pleurotus* were identified as better decolourizer of Poly R-478 dye among the nine species on solid medium. The selected four species (*P. flabellatus, P. ostreatus, P. sajor-caju and P. citrinopileatus*) were carried out for the five azo dye (Acid black, Congo red, Methyl orange, Methyl red and Phenol red) decolorization and activities of lignolytic (LiP, MnP and laccase) enzymes.

The higher activity of LiP was observed by *P. citrinopileatus* in liquid media supplemented with Acid black followed by *P. flabellatus* on Congo red, phenol red and methyl orange while the lowest activity was recorded on methyl Red by *P. citrinopileatus.*

*P. citrinopileatus* showed maximum MnP activity in liquid media containing methyl orange and Acid black followed by *P. flabellatus* on Phenol red and Congo red, whereas the maximum MnP activity was recorded on Congo red by *P .flabellatus*.

In methyl orange supplemented liquid media, *P. ostreatus* showed maximum laccase activity followed by *P. citrinopileatus* on Acid black, Phenol red and Methyl red and the minimum laccase activity in Congo red containing media.

Among the five dyes, methyl orange decolorization was faster and earlier i.e. 96.98% on the $20^{th}$ day on incubation by *P. citrinopileatus,* whereas remaining 4 dyes took 25 days for decolorization. 97.42% Congo red, 90.6 7% methyl red and 84.52% Acid black were decolourized by *P. flabellatus* letters and 93.33% phenol red was decolourized by *P. citrinopileatus* $25^{th}$ day of incubation.

The strain improvement program was started with the isolation of 12 monokaryons from each species of *Pleurotus* by the spore print generation. 48 monokaryons (homokaryons) were tested for mating response, further the monokaryons with similar mating behavior were stored out and were rearrange and classified into four distinct group (A1B1, A1B2, A2B1 and A2B2). A total of 16 group were obtained from four selected species and these group mated with each other to develop dikaryon (heterokaryons). Then these developed dikaryon were screened on agar plates containing 0.02% poly R-478. Four most promising dikaryons (heterokaryons) i.e. Pfo 6X9, Posc4X6, Poc9X6 and Pscc 8X3 were selected on the basis of higher growth rate (radial growth) and decolorization on potato dextrose agar containing my media containing screening dye.

After the selection of most promising dikaryons (heterokaryons), these were inoculated in liquid medium supplemented with poly R-478 at the concentration of 0.02%, 0.025% and 0.03% for the evaluation of tolerance level. At the higher concentration Poc 9X6 and Pfo 6X9 showed better decolourization, whereas less or no decolorization was found by Pscc 8X3 and Posc 4X6.

Different pH was studied for enzymes activity and decolorization of the earlier mentioned dyes. pH 5.5 facilitated dikaryons (heterokaryons) for the maximum activity of a LiP, MnP and laccase enzymes as well as dye decolorization in comparison to other pH.

The effect of age and concentration of inoculum were also studied. It was found that 5 ml of 10 days old culture support maximum activity of LiP and Laccase by Pfo 6X9 on Congo red and Poc 9X6 on methyl red. On contrary or this result 3ml of 10 days old culture of Poc 9X6 showed maximum MnP activity. In dye decolorization study 5 ml of 10 days old culture is effective for decolorization of all the dyes except Acid black which decolourized by 5 ml of 15 days old culture of improved dikaryon i.e. Poc 9X6.

Effect of surfactant example SDS (anionic) and tween-80 (cationic) on the enzyme activity and decolorization of the dye were investigated 0.1% tween-80 supported to dikaryon for maximum LiP and laccase activity on dye containing liquid medium while MnP showed maximum activity in the presence of 0.5 mM SDS by improved dikaryon. The maximum decolorization of dye (methyl red) by improved dikaryon was also found with 0.1% tween-80.

The increased LiP activity of improved dikaryon (heterokaryon) was observed in the Acid black containing media supplement with 2.0µM Tryptophan, whereas 2.0µM tyrosine assisted the maximum MnP and laccase activity by improve heterokaryon in the methyl orange in acid Black containing media respectively. The maximum phenol red decolorization was also found by improved dikaryon in the presence of 2.0µM tyrosine.

The effect of trace elements on the improved dikaryon (heterokaryons) for enzymes activities and dye decolorization were estimated during the present investigation. It was found that the lower concentration of Zn supported the maximum LIiP activity, while the MnP activity was higher in the presence of Mn at lower concentration. The laccase activity and decolourization of the dye was achieved by improved heterokaryon in the presence of Cu at lower concentration.

The main finding of the present study is that the improved dikaryon (heterokaryon) showed better lignolytic enzyme activities and decolorization of the above mentioned I in comparison to parent (*Pleurotus)* species. It was also found that all studied parameters i.e. pH, age and concentration of inoculum, surfactants, aromatic amino acid and trace elements markedly affect the enzyme activity and dye decolorization.

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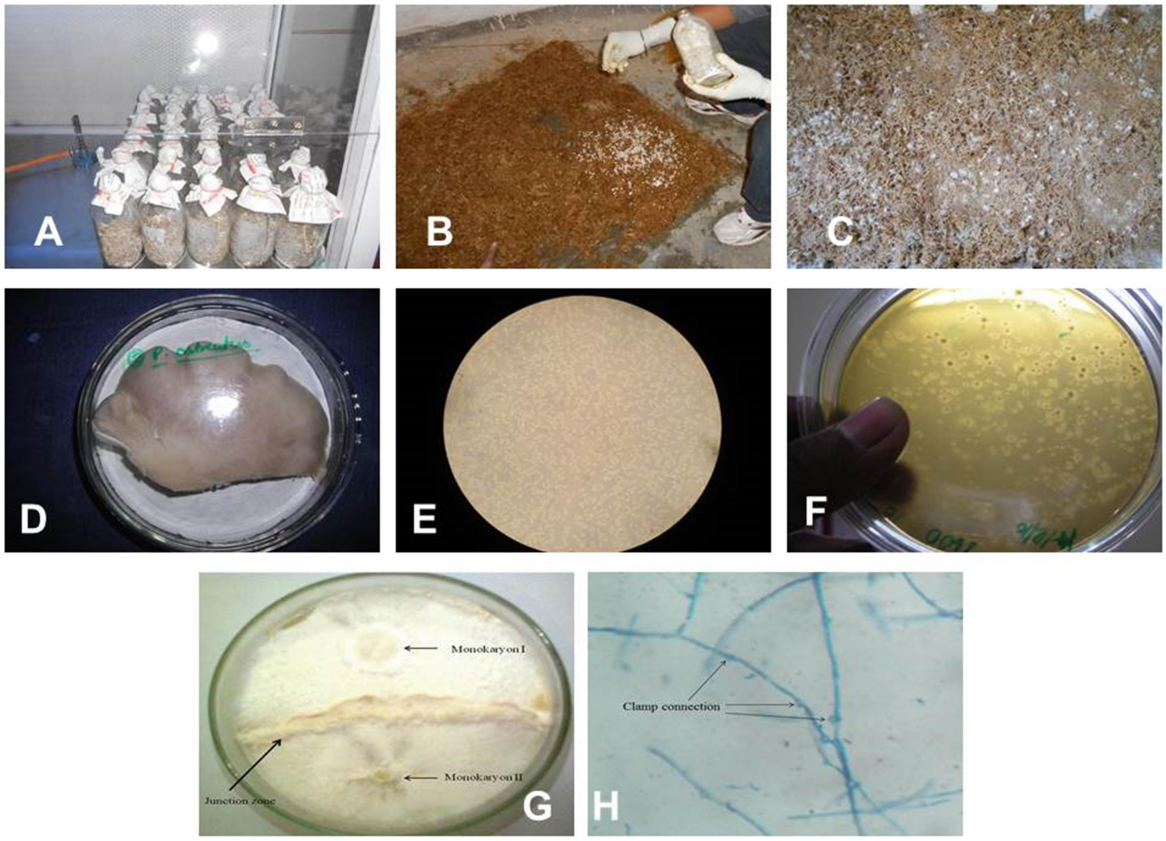


Figure 1. A- Spawn; B- Substrate preparation; C- Spawning; D- Spore print; E- Basidiospores; F- Single spore (monokaryons); G- Junction zone of two different monokaryons; H- Clamp connection formation.

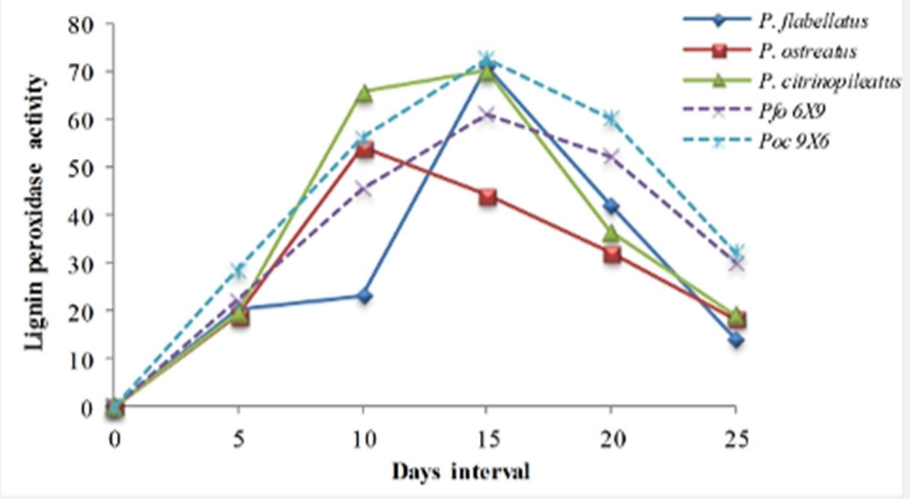


Figure 2. Lignin peroxidase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

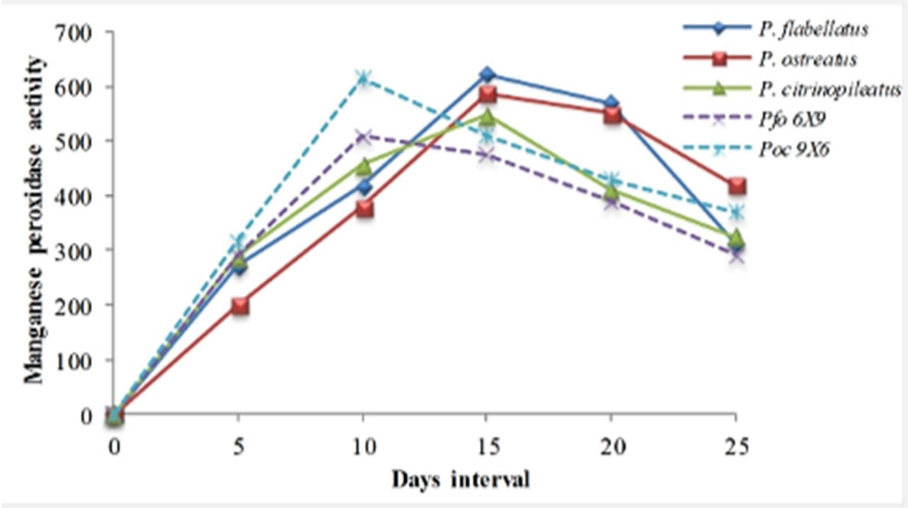


Figure 3. Manganese peroxidase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

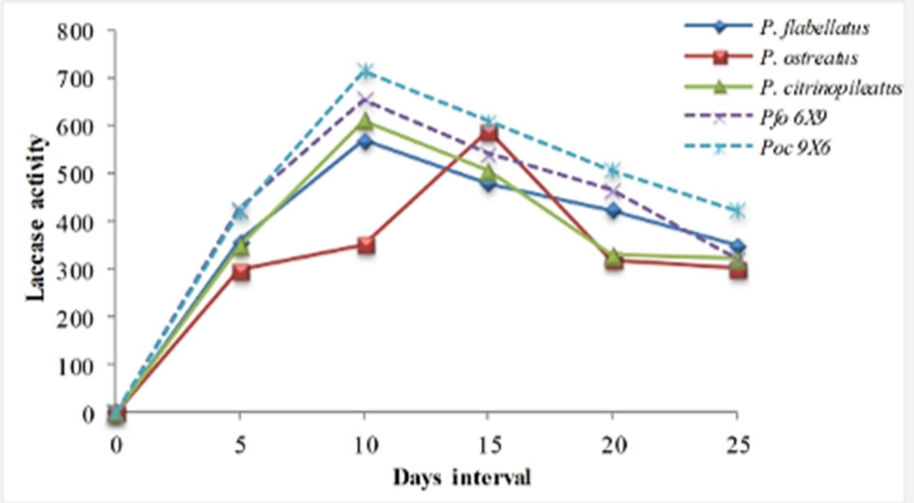


Figure 4. Laccase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

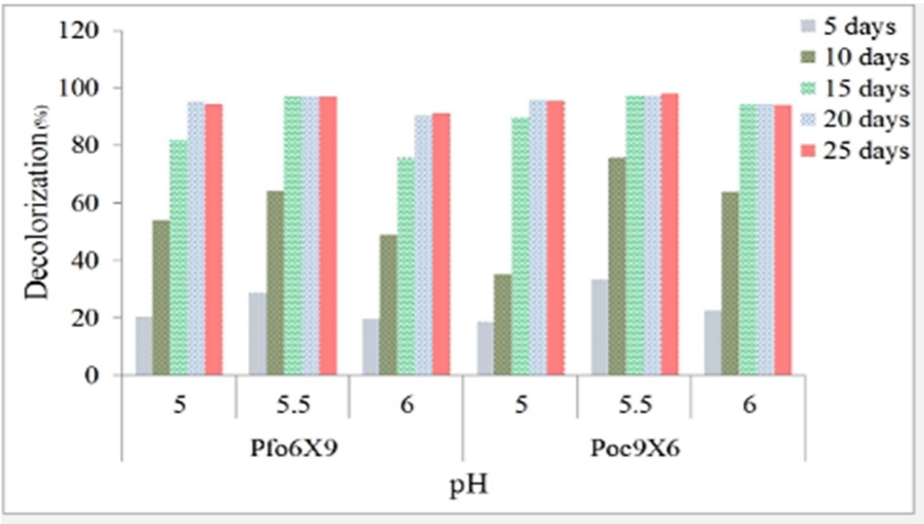


Figure 5. PSP decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different pH.

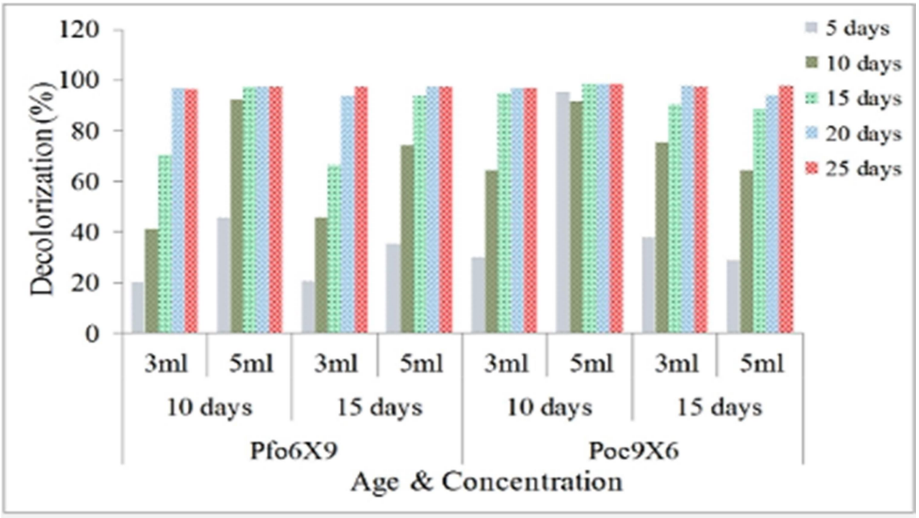


Figure 6. PSP decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different age and concentration.

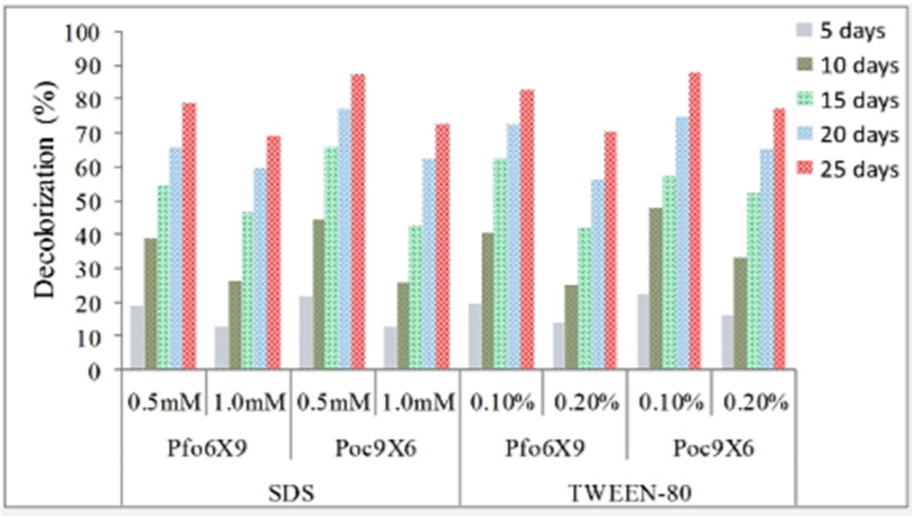


Figure 7. PSP decolorization by Pleurotus species and their basidiospore derived dikaryons (heterokaryons) at different concen-tration of SDS and Tween-80.