

Decolorization of Synthetic Textile Dye and Enzymes Production by Improved Strains of *Pleurotus* Species

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Abstract

In the present investigation the efficiency of three species of white rot fungi - *Pleurotus* and their improved dikaryons (heterokaryons) was assessed for decolorization of Phenol Red (Phenolsulfonphthalein or PSP) and production of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase enzymes. All the species of *Pleurotus* i.e. *P. flabellatus*, *P. ostreatus* and *P. citrinopileatus* decolorized PSP well. However, improved dikaryons Pfo 6X9 and Poc 9X6 decolorized the dye more effectively than three species of *Pleurotus*. The improved dikaryons also showed higher ligninolytic activity than the parental species. Poc 9X6 showed higher LiP (56.14 U), MnP (615.15 U) and Laccase (715.25 U) activity. In the present work different pH, age and concentration of inoculum and effect of surfactant i.e. sodium dodecyl sulphate (SDS) and Tween-80 were analyzed in order to determine the optimum ones to decolorize maximum concentration of dye. 5 ml of 10 days old culture on pH 5.5 and 0.1% Tween-80 supported maximum decolorization of PSP.

Keywords: Dye decolorization; Hyphal anastomosis; Ligninolytic enzymes; Phenol Red; *Pleurotus*

Abbreviations: %DD: Percent Dye Decolorization; LiP: Lignin Peroxidase; MnP: Manganese Peroxidase; PDA: Potato Dextrose Agar PSP: Phenolsulfonphthalene or Phenol Red; SDS: Sodium Dodecyl Sulphate; U: $\mu\text{m}/\text{ml}/\text{min}$; WRF: White Rot Fungi.

Introduction

A vast amount of dyes are produced annually with a variety of color and chemical structure around the world. These synthetic organic compounds with multiple aromatic rings either fused or connected covalently and modified with various hydrophilic functional group 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 byproducts 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 [7-9]. 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 which produces 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* 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°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°C in BOD incubator in cotton

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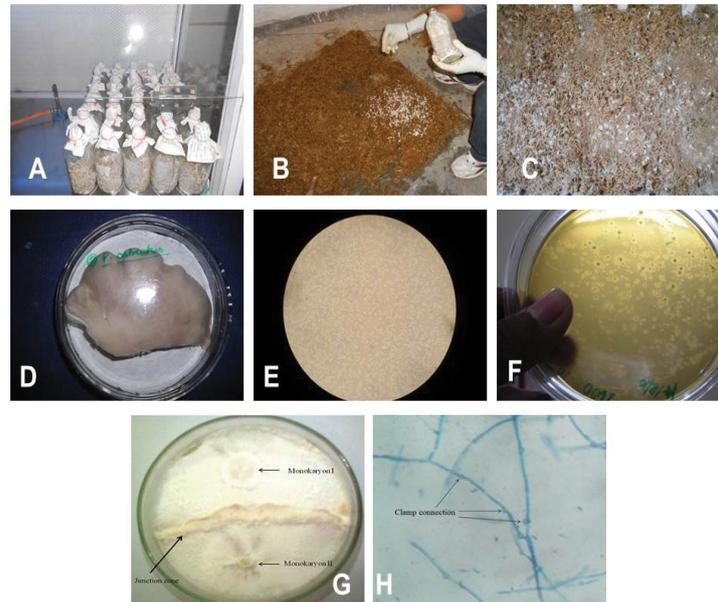


Figure 1: A-Spawn; B-Substrate preparation; C-Spawning; D-Spore print; E-Basidiospores; F-Single spore (monokaryons); G-Junction zone of two different monokaryons; H-Clamp connection formation.

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 (EC 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 λ_{max} 310 nm (41). One activity unit was defined as 1 μ mol 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 $MnSO_4$, 0.1 ml of 1 mM H_2O_2 , 0.25 ml of 1 mM guaiacol and 0.3 ml of crude enzymes. The oxidation of substrate at was followed spectrophotometrically at λ_{max} 465 nm [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 contains 1 ml of 1 mM guaiacol in 0.1 M sodium phosphate buffer (pH 6.0) and 1 ml crude enzyme solution was incubated at 30°C for 10 min. The oxidation was followed by the increase in absorbance at λ_{max} 495 nm [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 Figures 1A-1C.

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 1D). 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°C for their use in single spore isolation.

Germination and isolation of homokaryons: Paper bearing spores was cut into 2 × 2 cm size and suspended in 0.5% NaCl in 100 ml sterilized double distilled water and agitated at 150 rpm in orbital shaker for 2 hour to make uniform suspension (Figure 1E). The spore suspension further serially diluted up to 10^{-4} dilution from which 150 μ l 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°C in BOD incubator for one week. After germination of single spore (Figure 1F) 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°C in BOD incubator for further use.

Mating test: The mating compatibility between heterokaryotic cultures were performed in duel culture technique by placing actively growing mycelia (1 mm in diameter) of single spore cultures of above two strains approximately 1cm apart in the center of a 90 mm petriplate of potato dextrose agar (Figure 1G). Three replicates were used for each combination and arranged in a completely randomized design. In each step crosses were confirmed through clamp connection under 100 X magnification with cotton blue stain (Figure 1H). After the confirmation, a sample of mycelia was transferred to fresh agar medium for further examination of dye decolorization and enzymatic activities.

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°C. Dye disappearance was detected spectrophotometrically (Elico 164-SL) at λ_{max} 497 nm for PSP after 20th 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

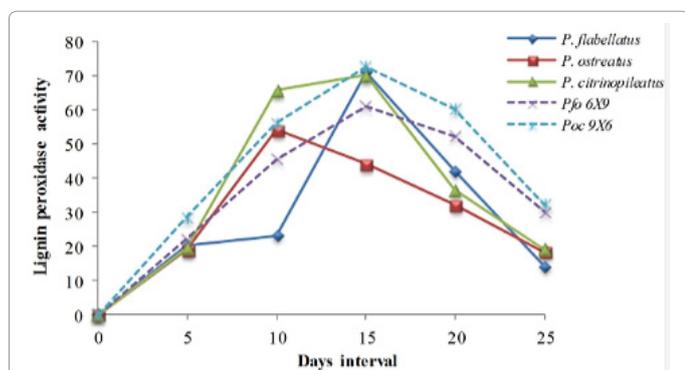


Figure 2: Lignin peroxidase activity ($\mu\text{M}/\text{ml}/\text{min}$) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

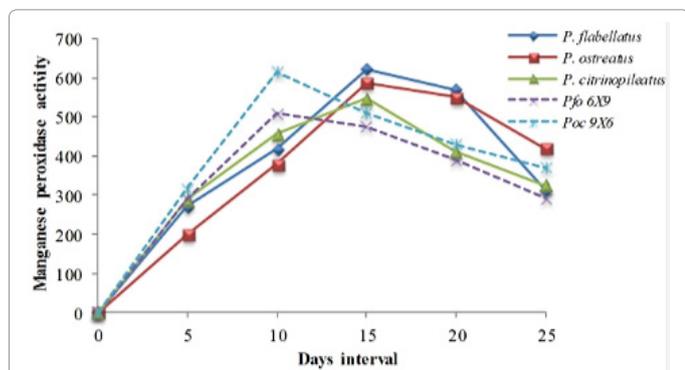


Figure 3: Manganese peroxidase activity ($\mu\text{M}/\text{ml}/\text{min}$) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

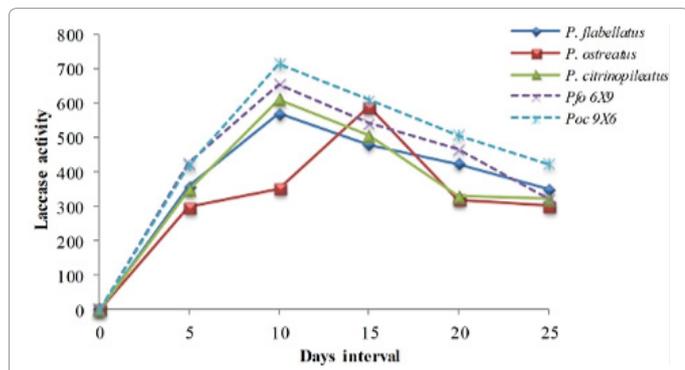


Figure 4: Laccase activity ($\mu\text{M}/\text{ml}/\text{min}$) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

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°C for 10 and 15 days. After the maximum growth of mycelia, homogenize suspension was made at 150 rpm in orbital shaker with the help of sterilized small glass pieces. The mycelial suspension was then inoculated in 100 ml Erlenmeyer flask, containing 30 ml dye containing broth medium at the concentration of 3 ml and 5 ml 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 Erlenmeyer 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°C for observing the dye decolorization [18].

Results

Ligninolytic enzymes

The lignin peroxidase activity of all the five species including basidiospore derived dikaryons is given in Figure 2. After 5 days of incubation Poc 9X6 showed 28.55U 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*.

Figure 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 10th 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 Figure 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 10th 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 phenol red decolorization

Effect of pH: Figure 5 shows the decolorization of PSP by *Pleurotus* species and improved dikaryons at pH 5.0, 5.5 and 6.0. 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: Figure 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.

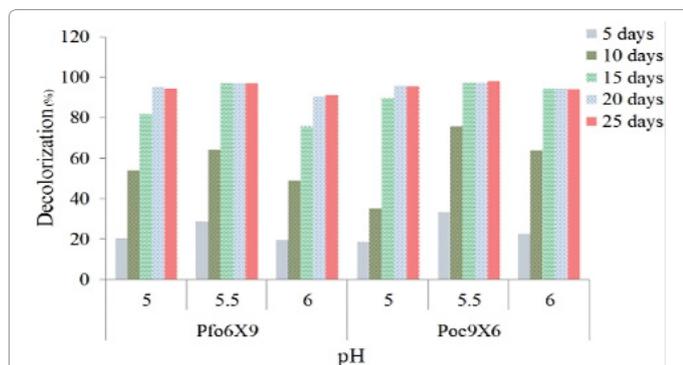


Figure 5: PSP decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different pH.

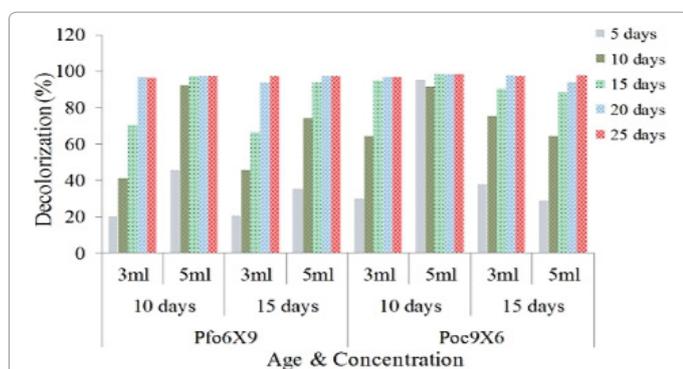


Figure 6: PSP decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different age and concentration.

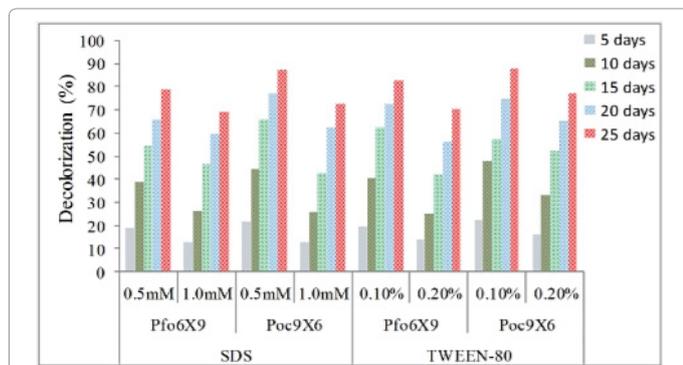


Figure 7: PSP decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different concentration of SDS and Tween-80.

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

Discussion

The lignin modifying enzymes (LMEs), i.e. lignin peroxidases (LiP, E.C. 1.11.1.14); manganese peroxidases (MnP, E.C. 1.11.1.13) and laccases (Lac, E.C.1.10.3.2), are produce by some WRF while, others 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]. Lignolytic 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]. 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. *Cerriopsis subvermispora*, *Dichotomitus squalens*, *Panus tigrinus*, *Rigidosporus lignosus*) do not excrete this enzyme [18,30-33]. The higher MnP activity was observed by improved dikaryons whereas, the parental (*Pleurotus*) species showed less MnP activity in PSP containing media. The basidiospore derived monokaryotic isolates is an efficient method of reaching higher variation in the production of MnP [34,35]. The principle role of Mn²⁺ to Mn³⁺, which then binds to an 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].

Decolorization 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 decolorization of Direct red by improved strains of *Pleurotus* species [41]. Dominguez et al. (6) re-ported 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 [41]. 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% Tween-80 supported highest MnP activity [37]. Tween-80 contains an oleat (an unsaturated fatty acid) which can peroxidized 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].

Conclusion

The three species of white rot fungi i.e. *Pleurotus flabellatus*, *P. ostreatus* and *P. citrinopileatus* can be used effectively and efficiently for dye decolorization and bioremediation of recalcitrant substances. However, the improved dikaryons Pfo 6X9 and Poc 9X6 of *Pleurotus* species prepared by crossing of basidiospores-derived monokaryons can decolorized the dye more effectively than three species of *Pleurotus*. Besides, improved strains also exhibited higher production of extracellular ligninolytic enzymes which have many industrial applications.

Acknowledgment

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