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Direct red decolorization and ligninolytic enzymes production by improved strains of *Pleurotus* using basidiospore derived monokaryons

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Abstract

In the present investigation the efficiency of three species of *Pleurotus* and their improved dikaryons (heterokaryons) was assessed for decolorization of direct red 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 the dye Direct Red well. However, 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 (76.27U), MnP (623.24U) and laccase activity (594.80U). 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 direct red dye.

Key words: Basidiospore, Dikaryons, Direct Red, Dye, Hyphal anastomosis, *Pleurotus*.

Introduction

Dyes are widely used by food, pharmaceutical, woolen, paper, metal, cosmetics and textile industries. During the dying process about 10-15% of the dyes used are released into the wastewater. The presence of these dyes used in the aqueous ecosystem is the cause of serious environmental and health concerns (1, 2). They are considered as xenobiotic compounds that are very recalcitrant, highly aromatic and having low biodegradability (3, 4).

Azo dyes are the largest group of synthetic dyes with extensive industrial applications due to their relatively simple synthesis and almost unlimited number and types of substituents. Azo dyes are a group of compounds characterized by the presence of one or more aromatic compounds with one or more azo (-N=N-) groups (5, 6). As these dyes are synthetic, they are generally toxic, mutagenic and resistant to biodegradation. Color of the dyes can create a significant environmental problem by affecting water transparency as well as aesthetic problems (7). The rate of natural degradation of recalcitrant compounds is very slow and it persists in environment for a longer period of time and magnified through the progression of trophic level. Physical and chemical treatments of wastewater containing dye residues are very expensive and generate large volumes of sludge and in certain cases they need chemical additives that, in turn, can be hazardous for the environment. Mycoremediation is a proper and green solution of eradication and reduction of the existing environmental pollutant such as synthetic dyes, heavy metals and xenobiotic compounds. Due to their capability to degrade recalcitrant compounds and resistant to toxic level of pollutants in comparison to bacteria, the potentialities of white rot fungi (WRF) gained attention in early 1990s (8). These fungi, mostly belonging to basidiomycetes, have arsenal of enzymes like Lignin peroxidase (LiP), Manganese peroxidase (MnP), Laccase and auxiliary enzymes like Glox, Polyphenol oxidase (PPO), Aryl alcohol oxidase (AAO) etc. which are capable of degrading lignin and related aromatic compounds.

The need of hours is not only to explore other new species of *Pleurotus* but also to improve the existing species through various breeding techniques like hyphal anastomosis and protoplast fusion. Scanty reports are available for dye decolorization and degradation by edible oyster mushroom, the *Pleurotus* species (9, 10, 11, 12, 13, 14, 15, 16, 17). The higher production of laccase and other lignolytic enzymes from dikaryons of *P. ostreatus* were obtained after crossing of relevant basidiospores derived monokaryons selected from the parental basidiospores population (18). Obtaining new strains through hybridization by protoplast fusion is tedious, expansive, time consuming and difficult in respect of the mushroom.

The present investigation was taken up with the aim to prepare the improved dikaryons (heterokaryons) of *Pleurotus* species by crossing of basidiospores- derived monokaryons avoiding protoplast fusion and mutagenesis, which can decolorize Direct Red and produce ligninolytic enzymes to maximum extent.

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 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 lignolytic 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 λ_{max} 310nm (6). One activity unit was defined as1 µmol 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 (A_{465}) (19).

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 (pH6.0) and 1ml of crude enzyme solution was incubated at 30^{0}C for 10min. The oxidation was followed by the increase in absorbance at 495nm. (20).

Fructification and basidioapore isolation

Cultivation

The method of spawn, substrate preparation and spawning were described in our earlier published paper (21, 22).

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. 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 100ml sterilized double distilled water and agitated at 150 rpm in orbitary shaker for 2 hour to make uniform suspension. 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 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 subcultured on PDA slants and incubated at 25^{0}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 (1mm 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 (Fig.1). 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 (Fig. 2). After the confirmation a sample of mycelia was transferred to fresh agar medium for further examination of dye decolorization and enzymatic activities.

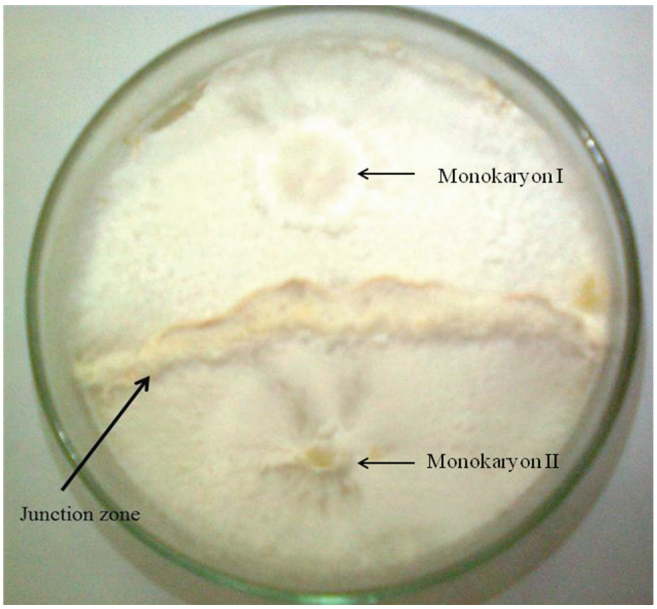

Figure 1. Thick barrage formed at junction zone of two monokaryons of *Pleurotus* species.

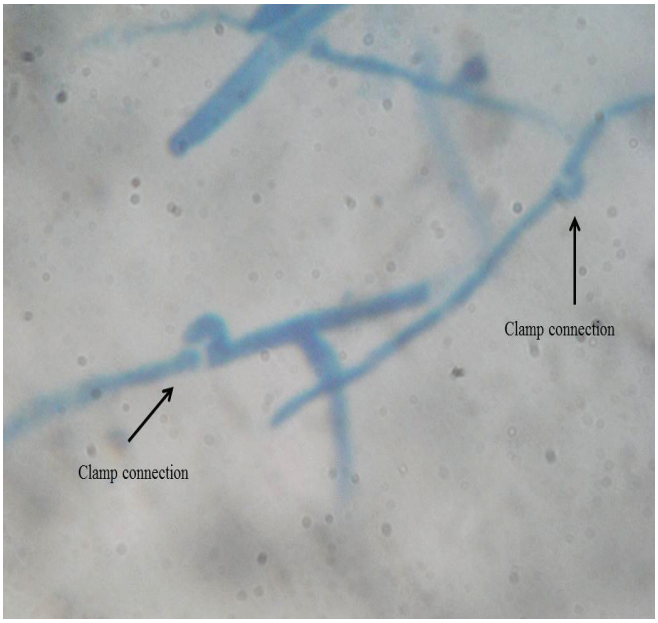

Figure 2. Clamp connection formation after hyphal anastomosis.

Decolorization studies in liquid media

The mycodecolorization experiments were done in potato dextrose broth medium supplemented with direct red 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 λ_{max} 497 nm for direct red dye after 20th days of incubation. Results were reported as the mean value of percent dye decolorization (%DD) for three replicates (23).

Optimization of parameter for direct red decolorization

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.

Age and Concentration of inoculums

Mycelial bits of 1mm in diameter were inoculated in 100 ml of potato dextrose broth medium in 250 ml Erlenmayer 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 were then inoculated in 100 ml Erlenmayer 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 decolourization (24).

Effect of Surfactants

Two types of surfactants - anionic surfactant i.e. Sodium dodecyl sulphate (SDS) and nonionic surfactant i.e. Tween-80 were used for 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 (25).

Results

Ligninolytic enzymes

Lignin peroxidase activity of *Pleurotus* species and their basidiospore derived dikaryons is given in Fig.3. After 5 days of incubation *P. flabellatus* showed 48.2 U LiP activity whereas, *P. ostreatus*, *P. citrinopileatus*, Pfo 6X9 and Poc 9X6 showed 11.4, 14.13, 18.42, and 26.80 U respectively. During time course of culturing, basidiospore derived dikaryon Poc 9X6 showed maximum LiP activity i.e., 76.27 U on 10th day of incubation, followed by *P. flabellatus, P. citrinopileatus*, *P. ostreatus* and Pfo 6X9.

Fig. 4 shows Manganese peroxidase activity of *Pleurotus* species and their basidiospore derived dikaryons. After 5 days *P. flabellatus* showed 273.73 U LiP activity whereas, *P. ostreatus*, *P. citrinopileatus*, Pfo 6X9 and Poc 9X6 showed 218.07, 181.27, 240.34, and 212.46 U respectively. After 15 days, improved dikaryon Poc 9X6 showed maximum LiP activity i.e., 623.24 U, followed by Pfo 6X9, *P. flabellatus, P. ostreatus* and *P. citrinopileatus*.

Laccase activity of *Pleurotus* species and their basidiospore derived dikaryons is presented in Fig. 5. Among all five species including dikaryons, after 5 days of incubation Poc 9X6 achieved 517.14 U LiP activity whereas, *P. flabellatus, P. ostreatus*, *P. citrinopileatus* and Pfo 6X9 showed 292.47, 304.33, 377.93 and 408.82 U respectively. On 10th day of incubation dikaryon Poc

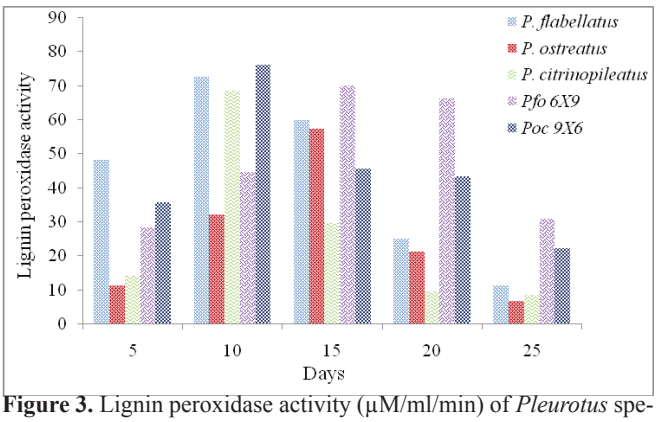

Figure 3. Lignin peroxidase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

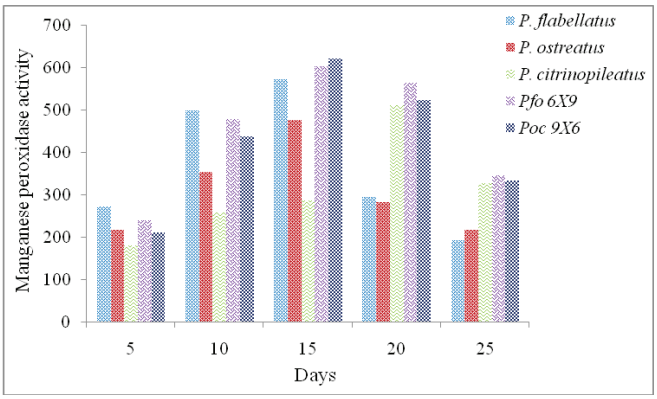

Figure 4. Manganese peroxidase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

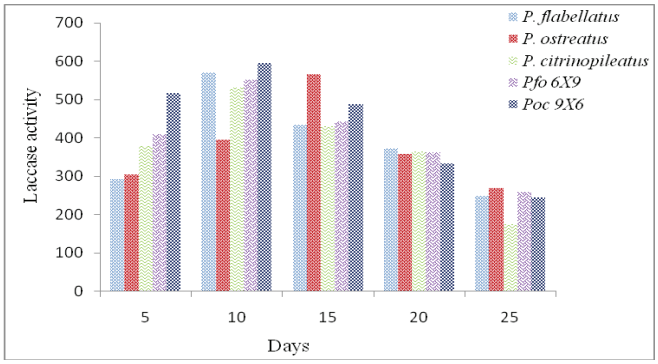

Figure 5. Laccase activity (µM/ml/min) of *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons).

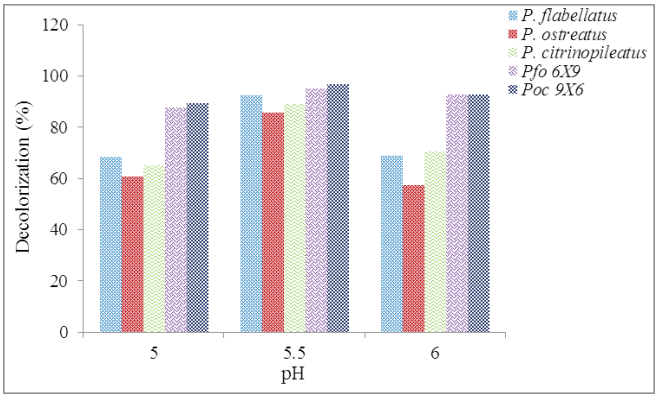

Figure 6. Direct red decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different pH.

9X6 showed maximum LiP activity i.e., 594.80 U, followed by *P. flabellatus,* Pfo 6X9, *P. citrinopileatus* and *P. ostreatus*.

Optimization of parameter for direct red decolorization

pH

Fig. 6 illustrates the decolorization of direct red 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 Pfo 6X9.

Age and concentration of inoculum

The effect of mycelial age and concentration of all *Pleurotus* species and improved dikaryons for direct red decolorization is depicted in Fig.7. The maximum decolorization was gained by 5 ml 10 days old culture of Poc 9X6 followed by others.

Surfactant

Fig. 8 shows the effect of SDS and Tween-80 on *Pleurotus* species and improved dikaryons for direct red decolorization. 0.1% Tween-80 and 0.5 mM SDS supported to Poc 9X6 for maximum decolorization of direct red.

Discussion

White rot fungi are key regulators of the global C-cycle. Their 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 directly involved not only in the degradation of lignin in their natural lignocellulosic substrates

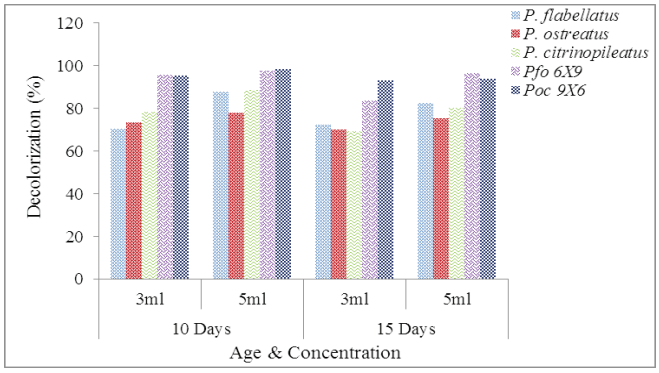

Figure 7. Direct red decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different age and concentration.

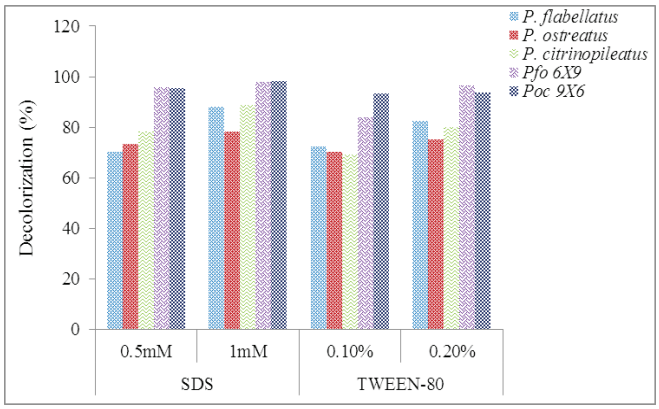

Figure 8. Direct red decolorization by *Pleurotus* species and their basidiospore derived dikaryons (heterokaryons) at different concentration of SDS and Tween-80.

(26, 27) but also in the degradation of various xenobiotic compounds (28, 29, 30) including dyes (31, 32, 33, 34, 35). Some white rot fungi produce all three lignin modifying enzymes (LME) while others produce only one or two of them (27). There are two major families of ligninolytic enzymes which are involved in lignolysis: peroxidases and laccases (35, 36, 37). 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 (38). Among the various white rot fungi, *Pleurotus* species have been reported the produce all the three modifying enzymes, which play a vital role in biodegradation and bioremediation (21, 22, 39). Lignolytic enzymes are produced in the initial stage while, cellulolytic and xylanolytic enzymes are produced in the later stage of growth of *Pleurotus* species (22, 40).

LiP is capable of oxidizing a variety of xenobiotic compounds, including polycyclic aromatic hydrocarbons, polychlorinated phenols, nitroaromatics, and azo dyes (41). The fungal LiP has been repeatedly implicated in the bleach of a diverse range of synthetic dyes (42, 43, 44). The maximum MnP production by isolates was higher than that by the parental strain (45). Some workers also showed that the basidiospore derived monokaryotic isolates is an efficient method of reaching higher variation in the production of lignolytic enzymes (46, 47). 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 (18, 45).

Baskaran and Dhansekar, reported pH 5.8 as best decolorization pH for all the dyes by *P. ostreatus* when the experiment were conducting at different initial pH ranging from 4.2 to 7.4 (48). Dominguez et al. (49) reported that, pH 4.5 supported higher peroxidase activity by *P. chrysosporium* on media containing Poly R-478. Singh et al. observed that, twelve days old culture of *Pleurotus flabellatus* showed the maximum enzymatic activity, that is, 915.7 U/mL and 769.2 U/mL of laccase and manganese peroxidase, respectively (50). Park et al. recorded that, 8 ml of mycelia suspension of 4 days old culture of white rot fungi was effective for decolorization of Acid yellow 99, Acid blue 300, and Acid red 114 (24). 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* (51). Unsaturated fatty acids (also present in Tween-80 as oleate) can peroxidized by MnP and the oxidants so generate could participate in organo-pollutant degradation by fungal culture (52, 53). The effect of five non-ionic surfactants had positive effect on decolorization of RO16 in both static and agitated culture of *I. lacteus* (54).

Other articles in this theme issue include references (55-70).

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