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Decolourization of azo, heterocyclic and reactive dyes using spent mycelium substrate of *Hypsizygus ulmarius*

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Abstract

Cultivation of *Hypsizygus ulmarius* to generate spent mycelium substrate (SMS) for dye decolourization gave better yield, biological efficiency, fruit width and moisture content (145 gm, 33%, 4 cm, 91%) on paddy straw as compared to coconut husk (59 gm, 21%, 3 cm, 90%). Solid-phase decolourization of Congo red (Azo dye) and Methylene blue (Heterocyclic dye) showed that maximum decolourization (3.31), measured as Decolourization Index, occurred at dye concentration of 25 mg l^{-1}, while in Solochrome black (Reactive dye), it was at 100 mg l^{-1} (1.7). Time taken for maximum decolourization was 10 days in Congo red and Solochrome black; 20 days in Methylene blue. Decolourization Index was maximum in Methylene blue (3.1), followed by Congo red (1.9) and Solochrome black (1.2). Liquid-phase decolourization of Methylene blue and Solochrome black showed that maximum decolourization (62.5%) measured as percent decolourization occurred at 25 mg l^{-1}, while it was at 50 mg l^{-1} (36%) for Congo red. Time taken for maximum decolourization for all three dyes was 10 days. During this period, the percent decolourization was maximum in Methylene blue (91.3%), followed by Solochrome black (82.2%) and Congo red (79.7%). Decolourization potential in solid-phase was observed till 100 mg l^{-1} and day 25 for all the three dyes, however, in liquid-phase it was observed till 50 mg l^{-1} and day 20 for Congo red, 75 mg l^{-1} and day 10 for Solochrome black, 100 mg l^{-1} and day 20 for Methylene blue. Maximum laccase was produced on day 25 during decolourization of 25 mg l^{-1} Congo red, while maximum Manganese peroxidase was noted on day 20 at 50 mg l^{-1} Congo red.

Key words

Azo dye, Heterocyclic dye, *Hypsizygus ulmarius*, Reactive dye, Spent mycelium substrate

Introduction

Mushroom cultivation is an environmental-friendly way of producing vegetarian protein and disposing agricultural wastes (Sarker *et al.,* 2008). Oyster mushrooms have enzymes such as cellulases, ligninases and xylanases that are capable of breaking down complex chemicals and plant matter, occurring as agricultural wastes (Baysal *et al.* 2003). With their nutritional value and high productivity, mushrooms can alleviate malnutrition in the rural poor (Ramabadran 2000), and are also active against hypercholesterolemia, hypertension, diabetes, cancer and various infections (Alam *et al.,* 2007). Spent mycelium substrate (SMS) is by-product of mushroom cultivation, consisting of partially degraded substrate and fungal mycelia capable of secreting enzymes. For every kilogram mushroom cultivated, approximately 5 kg of SMS is generated (Semple et al. 2001). Proper disposal of SMS is a challenging task due to its bulky nature; traditionally it is incinerated or buried (Ahlawat and Singh, 2011).

Mushroom industry is facing pressure from regulatory agencies to use it in a more environmental friendly manner (viz.) in bioremediation (i.e.) the use of SMS in dye decolourization, which was reported for *Pleurotus sps.* (Neelamegam 2004), or the use of SMS as bio fertilizer, as it is a rich source of carbon, nitrogen and other elements (Tallapragada *et al.,* 2011, Chorover *et al.,* 2000). SMS contains a rich and varied microflora, which have the ability to breakdown organic and inorganic xenobiotics in soil (Ahlawat, 2010). The role of extracellular ligninolytic enzymes and microbes from SMS of *Pleurotus florida*, *P. ostreatus, P.*

flabellatus and *P. sajor-caju* have been evaluated for their use in dye decolourization (Ahlawat 2006, Faraco *et al.,* 2009).

Azo dyes are used in textile, pharmaceutical, cosmetic and food industries. During processing, nearly 40% of the used dye is released in wastewater. This affects the aesthetics, transparency and dissolution of oxygen in water resulting in toxicity (Ali *et al.,* 2008). Dyes even at very low concentration (<1 mg l^{-1}) in the effluent are visible and undesirable, especially red colour (Forgacs and Oros, 2004).

Reactive dyes are significant because of their bright colour and low energy consumption during application (Aksu 2005). When they are present in exhausted dye baths and rinsing water, they are not recyclable or biodegradable (Borchert and Libra 2001). Dyes can cause allergic dermatitis, skin irritation, cancer and mutations in humans (Inbaraj *et al.,* 2002).

Presently, dyes are removed from effluents by physico-chemical methods which are costly and cause accumulation of sludge. There is a need to find alternative treatments like bioremediation which is both process-effective and cost-effective (Gupta *et al.,* 2011). Azo dyes can be decolourized by fungi, especially white-rot fungi (Kodam *et al.,* 2005). Microbial decolourization involves combination of oxidative and reductive steps. Oxidation is brought about by enzymes like peroxidases and laccases and usually found in these fungi for lignin breakdown (Gomare and Govindwar 2009).

The present study involved a preliminary assessment of solid and liquid phase dye decolourization by SMS of novel edible oyster mushroom, *Hypsizygus ulmarius*. Cultivation of *H.ulmarius* on two common agricultural wastes (viz.) paddy straw and coconut husk was carried out to compare biological efficiency of the substrates and to generate spent substrate for decolourization.

Materials and Methods

The spawn of oyster mushroom *H.ulmarius* was procured from IIHR, Bangalore, cultured on Mandel and Weber's modified agar (Mandels and Weber 1969), stored at 4°C and sub-cultured every three months. Pure culture was used for solid-phase decolourization, while the SMS from cultivation for liquid-phase study. Three different class of dyes studied were azo (Congo red), heterocyclic (Methylene blue) and reactive (Solochrome black). Concentrations of dyes tested were 25-100 mg l^{-1} and was studied for a time period of 25 days.

Solid phase decolourization : Fungal discs, 5-6 mm in diameter, were inoculated in petri plates containing Mandel and Weber's modified agar and the dyes, while un-inoculated plates were used as controls. The plates were incubated at 25°C for 25 days. Mycelial diameter and Decolourization diameter were measured on 10, 15, 20 and 25 day. Decolourization capacity was expressed as decolourization Index and calculated by the following formula:

$$D.I. = D.D. / M.D. (cm).$$

Liquid phase decolourization : For study liquid phase SMS of *H.ulmarius* was used. To generate SMS, the spawn of *H.ulmarius* was cultivated by solid-state fermentation, in clear polythene bags using paddy straw or coconut husk as substrates (Chang *et al.,* 1981).

Flasks containing Mandel and Weber's modified broth and dye were inoculated with 1 gm dried SMS, while un-inoculated flasks were used as controls. The flasks were incubated at 25°C for 25 days. Initial absorbance and subsequent periodic absorbance readings were noted on 10, 15, 20 and 25 day. For estimation, 2 ml aliquot was pipetted out, centrifuged for 2 min at 4000 rpm and maximum absorbance was read at 497, 644 and 618 nm for Congo red, Methylene blue and Solochrome black, respectively. Decolourization was calculated as:

$$\% \text{ decolourization} = [(A_o - A) / A_o] \times 100$$

where A_o is initial absorbance and A is periodic absorbance on that particular day.

Cultivation of *Hypsizygus ulmarius* : Crushed rice straw was used for cultivation. Straw was cut in to 2-6 cm pieces, soaked overnight and steam sterilized, for 1 hr at 65°C, while coconut husk was separated and soaked for 5-6 hrs, then steam sterilized as above. The amount of spawn used to inoculate the substrate was 5% of its total weight (50 gm spawn for 1 kg substrate). The pasteurized substrate was spawned and filled into clear perforated polyethylene bags; incubated at 23-25°C for 12 to 14 days. Mushrooms started to form around the edges of bag perforations and were approximately harvested after 3 to 4 weeks. After spawning, the bags were moved to a room harving temperature 18–20°C and relative humidity 95-98%. The first 12-21 days were completed without artificial lighting. At the end of spawn run, 4 hrs of light was provided daily by fluorescent bulbs. At the time of pinning (mushroom formation), sufficient fresh air was introduced to lower CO_2 level.

Nutritional analysis of mushrooms : The total yield was estimated by weighing the fruits, while width of fruit and length of stipe was measured using thread and graduated ruler. Biological efficiency is the yield of mushroom per kg of substrate on dry weight basis, and was calculated as % B.E = Fresh weight of mushroom / dry weight of substrate x 100 (Chang *et al.,* 1981). For estimating moisture content, the fresh fruit was weighed (W_1 gm), then dried in a hot-air oven at 50°C for 24 hrs and weighed again (W_2 gm). Percent moisture content was calculated as per Chang *et al.,* 1981.

Ligninolytic enzyme assay : Laccase activity was measured by oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic

acid) or ABTS at 5 mM. Substrate was dissolved in 2.4 ml sodium acetate buffer (0.1 M, pH 5) and 100 µl of culture filtrate added. The mixture was incubated at 30°C for 2 minutes and absorbance measured at 495 nm (Bourbounnais *et al.,* 1995).

Manganese peroxidise activity was measured by oxidation of Mn(II) to Mn(III), using 2.5 ml of phenol red (0.01%) and $MnSO_4$ (0.1 mM) in sodium succinate buffer (0.1 M) as substrate. A reaction mixture containing 2.5 ml of substrate and 200 µl of culture filtrate was prepared; reaction was initiated by addition of H_2O_2 (0.1 mM). After incubating for 2 min at 30ºC, reaction was stopped by addition of 5 M NaOH. Absorbance was measured at 610 nm (Glen and Gold 1985). The statistical analysis was done using Microsoft Excel.

Results and Discussion

Analysis of fruits after harvest showed (Table 1) that total yield, biological efficiency and fruit width was higher when cultivated on in paddy straw (145 gm, 33%, 4 cm) than coconut husk (59 gm, 21%, 3 cm); however stipe length was greater in coconut husk (3 cm) as compared to paddy straw (2 cm). Stipe length was highest in mushroom flushes produced on sawdust and coconut husk in the case of *Pleurotus sajor-caju* (Nurudeen *et al.,* 2013). Moisture content was slightly higher (91%) in straw than husk (90%).

Paddy straw is the preferred substrate for cultivation. However, coconut husk can be used as an alternative or supplement to paddy straw. Rice straw, cotton waste, coir, baggase and banana leaves are good substrates for growing oyster mushrooms (Belewu and Belewu, 2005). Yield and quality obtained depends on their C: N ratio and composition of vitamins, phytohormones, macro and microelements present (Adenipekun and Gbolagade, 2006). The natural substrates used for cultivation of *H. ulmarius* are paddy straw, wheat straw and millet straw. Coconut husk was a novel substrate that was used in this study. Both substrates are cost-effective and available in large quantities. In addition, use of these agricultural wastes in mushroom cultivation provides a solution for their disposal.

Solid-phase decolourization studies showed (Fig. 1a) that the potential for decolourization occurred in all three dyes till concentration of 100 mg l^{-1} and throughout the entire study period of 25 days. Maximum decolourization, calculated as Decolourization Index, was observed in methylene blue (3.05), followed by Congo red (1.85) and Solochrome black (1.207). This meant that decolourization diameter was relatively higher in Methylene blue with respect to mycelial diameter, and indicated that *H.ulmarius* decolourized Methylene blue to greatest extent. Indigo dye has been degraded by *Phellinus gilvus, Pleurotus sajor-caju* and *Pycnoporus sanguineus* (Balan and Monteiro, 2001); however another study showed that *Irpex lacteus* did not exhibit good decolourization potential for methylene blue (Novotny *et al.,* 2004).

Table 1 : Cultivation of *Hypsizygus ulmarius* on two common agricultural wastes

Parameters/ Substrate used	Yield (gm)	Biological efficiency (%)	Moisture content (%)	Fruit width (cm)	Stipe length (cm)
Paddy straw	145.1	33	91.3	3.5	2
Coconut husk	59.4	20.5	90.2	2.7	3

Congo red exhibited maximum decolourization at concentration of 25 mg l^{-1} (2.664), which further decreased till 100 mg l^{-1} (1.145). Studies have shown (Fig. 1b) that as the concentrations of Congo red increased, biosorption capability of *Trametes versicolor* decreased (Binupriya *et al.,* 2007). From Fig. 1a, time period of maximum decolourization was 10 days.

In Methylene blue, maximum decolourization was noted at 25 mg l^{-1} (3.962) and steadily reduced to 1.595 by 100 mg l^{-1} (Fig. 1b). Time taken for achieving maximum decolourization was, however, extended to 20 days (Fig. 1a). This rise of decolourization capability observed towards the end of the cycle was possibly due to carbon and nitrogen depletion in the medium by that time (Galhaup *et al.,* 2002). Time needed for complete decolourization for two dyes, viz. anthraquinone dye RBBR and polymeric dye Poly R-478 was 20 and 16 days, respectively, in case of *P. ostreatus*.

Solochrome black gave maximum decolourization at 100 mg l^{-1} (1.654). Decolourization index increased marginally from 1.085 at 25 mg l^{-1} and was nearly constant from 25-100 mg l^{-1} (Fig. 1b). Ligninase functions better in nitrogen-depleted conditions, especially for higher concentrations. Nitrogen-sufficient cultures typically exhibit better growth immediately and for few days after inoculation. Increased growth results in carbon depletion, especially glucose, by 4th day. Carbon starvation induces idiophasic metabolism and production of lignin peroxidase (Baldrian, 2004).

Maximum time taken for decolourization for Solochrome black was 10 days, after which decolourization index slightly reduced but remained fairly constant (Fig. 1a). Rate of decolourization usually varied with the chemical structure of dye and secretion of extracellular laccase and manganese peroxidase. Increase of these enzymes always accelerates decolourization (Novotny *et al.,* 2004).

In all three dyes decolourization occurred till 100 mg l^{-1} and day 25 . Acid Violet 19 was decolourized upto 250 mg l^{-1} by *Pseudomonas aeruginosa BCH*. Initial dye concentration, pH and temperature are important factors that determine the extent of decolourization (Jadhav *et al.,* 2012).

Liquid-phase decolourization studies showed (Fig. 2a) that maximum decolourization accured till day 10. Percent

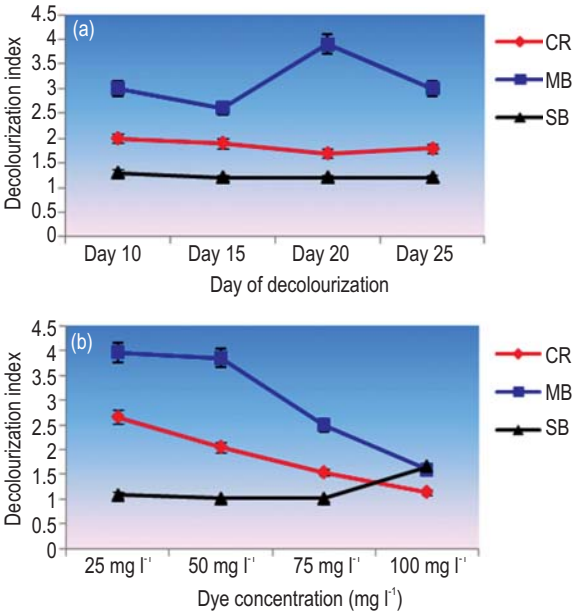

Fig. 1 : Efficiency of solid-phase decolourization of Congo red (CR), Methylene blue (MB) and Solochrome black (SR) by *H. ulmarius* (a) days and (b) at varying dye concentrations

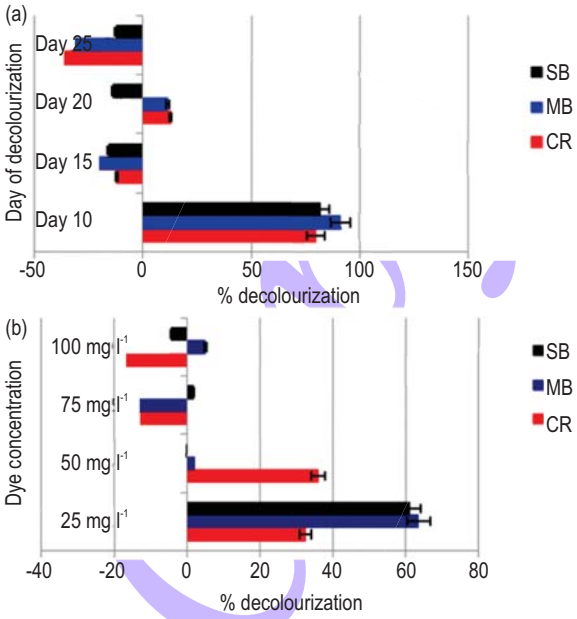

Fig. 2 : Efficiency of liquid-phase decolourization of three dyes Congo red (CR), Methylene blue (MB) and Solochrome black (SR) by *H. ulmarius* (a) days and (b) at varying dye concentrations

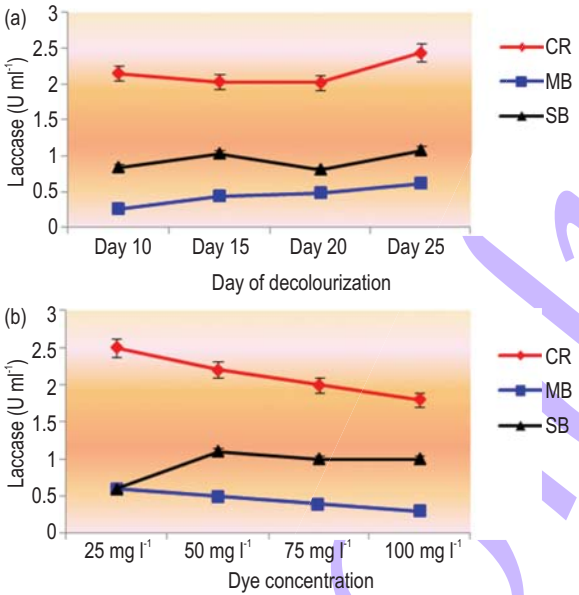

Fig. 3 : Laccase production during decolourization of Congo red (CR), Methylene blue (MB) and Solochrome black (SR) by *H. ulmarius* (a) days and (b) at varying dye concentrations

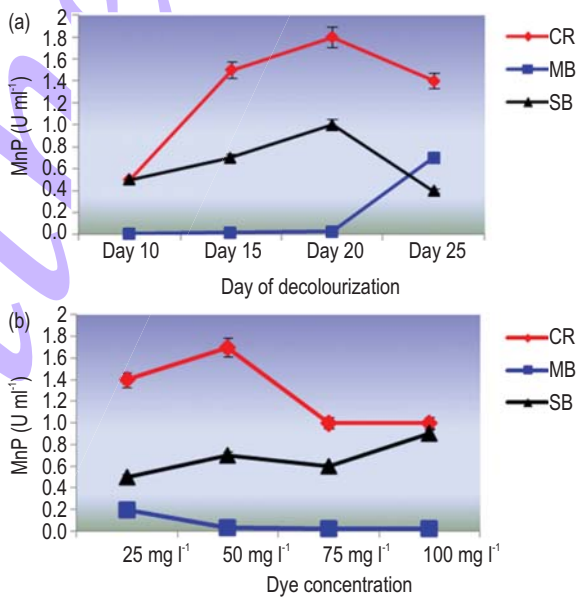

Fig. 4 : Manganese peroxidase production during decolourization of Congo red (CR), Methylene blue (MB) and Solochrome black (SR) by *H. ulmarius* (a) days and (b) at varying dye concentrations

decolourization was observed maximum in Methylene blue (91.3%), followed by Solochrome black (82.2%) and Congo red (79.7%). Decolourization potential was observed till 50 mg l^{-1} and day 20 for Congo red, 75 mg l^{-1} and day 10 for Solochrome black, 100 mg l^{-1} and day 20 for Methylene blue. Ravikumar *et al.* (2013) showed that Congo red decolourized upto 62% using purified

laccase from *H.ulmarius*. In the present study both Methylene blue and Solochrome black exhibited maximum decolourization at 25 mg l^{-1} (63.6% and 61.3%, respectively), after which it steeply decreased to 2.15% in Methylene blue and 1.8% in Solochrome black. Congo red showed decolourization of 32.6% at 25 mg l^{-1} and 36% at 50 mg l^{-1}, after which it became, almost nil (Fig. 2b). About 22% removal of colour containing azo dyes was observed by day 9, with *Clitocybula dusenii* (Buchon and Agathos 2002), while Lanaset Brown B was decolourized upto 96% by *Halobacillus sps.* C-22 (Demirci *et al.,* 2011).

Maximum laccase was produced on day 25 during decolourization of 25 mg l^{-1} Congo red (Fig. 3a, 3b), while maximum Manganese peroxidase was found on day 20 and 50 mg l^{-1} Congo red (Fig. 4a, 4b). Sathishkumar *et al.,* 2013 reported that Remazol Brilliant blue R was decolourized upto 46% by laccase of *P. florida* in 10 min, and at maximum decolourization potential of 87%, the enzyme concentration was 1.87 U ml^{-1}. Decomposition of Indigo carmine by *Phanerochaete chrysosporium* showed that ligninolytic enzymes were responsible for decolourization (Podgornik *et al.,* 2001). Production of ligninolytic enzymes is affected by media composition, C : N ratio, pH, temperature, rate of agitation of cultures and presence of certain aromatic compounds (Arora and Gill 2001). Decolourization of the azo dye, Congo red, reached a maximum of 95% when manganese peroxidase increased 400-fold (Novotny et al. 2004).

Conventional physico-chemical methods used for decolourization of dyes are effective but are costly (De Moraes *et al.,* 2000). Ligninolytic fungi can degrade a broad spectrum of structurally different dyes due to their highly oxidative and non-specific enzyme systems, developed for lignin mineralization (Saparrat and Guillen, 2005). Physical adsorption onto the surface of spent mycelium followed by biochemical breakdown of dye molecules by enzymes are the usual mechanisms (Zumriye and Karabayir, 2008).

Oxygen initiates decolourization and activates the catalytic system. Possibly, there is a single catalytic system responsible for degradation of both lignin and dyes; however, specific reactions are dependent on the physico-chemical properties of substrates. Dyes like Acid Navy blue and Methylene blue were adsorbed onto the biomass of a closely related strain of *Aspergillus lentulus*, produced by using corn cob through solid-state fermentation (Kaushik *et al.,* 2013). Using micro organisms makes the process relatively inexpensive and the end products are non-toxic (Stolz 2001).

Thus, it can be concluded that oyster mushroom, *H.ulmarius* can be better cultivated on paddy straw than coconut husk. The spent mycelium substrate (SMS) from cultivation can be effectively used for decolourization of all the three dyes studied. The enzymes secreted during decolourization belonged to ligninase complex, of which laccase is the most effective; laccase functioned effectively for decolourization of Congo red.

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