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A comparative study on decolorization of reactive azo and indigoid dyes by free/immobilized pellets of *Trametes versicolor* and *Funalia trogii*

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Abstract

The objective of the present study was to investigate decolorization of Acid Blue 74 and Reactive Blue 198 dyes by free and immobilized white rot fungal pellets in order to confirm the possibility of practical application *via* repeated-batch cultivation. Decolorization studies were conducted using free pellets (FP), fungal cells immobilized on activated carbon (IFCAC) and pinewood (IFCP), and also fungal cells entrapped in alginate beads (FCEAB). No additional nitrogen and carbon source was used and high decolorization rates were achieved in only dye-contained media without pH adjustment. Acid Blue 74 was decolorized 96 and 94% within 2 hr by *Trametes versicolor* and *Funalia trogii* free pellets, respectively. These values were 87 and 84% for Reactive Blue 198, in this respect. Immobilization of fungal cells on pinewood increased the usability of pellets and the average decolorization efficiency of both dyes. The micro environment changed in the presence of pinewood and increased the stability of immobilized pellets. Decolorization was performed rapidly and efficiently. Laccase activity enhanced with availability of pinewood, and high laccase production with *F. trogii* was obtained. After separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the molecular weight of *T. versicolor* and *F. trogii* laccase bands was determined 64 and 61 kDa approximately. Green bands were obtained by the activity staining process with laccase substrate (ABTS) after gel renaturation step.

Key words

Decolorization, Immobilization, Laccase, Textile dye, White rot fungi

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Introduction

Different dyestuffs have highly varying chemical characteristics and are selected according to the material to be dyed. Therefore, the composition of dyeing effluent varies based on textile products (O'Neill *et al.*, 1999). Majority of synthetic dyes currently used in textile industry are azo dyes (Forgacs *et al.*, 2004). Reactive azo and vat dyes are largely employed on cellulosic fibers like cotton (Manu and Chaudhari, 2003). In textile industry, large amount of complex dyestuff are discharged into lakes and rivers every day, leaching to contamination of groundwater and rivers (Mohorcic *et al.*, 2006; Stolz, 2001). Due to low biodegradability, they cause serious environmental pollution (Eichlerova *et al.*, 2007). The major problem encountered with use of synthetic dyes is their complex aromatic molecular structure which is designed to be resistant to physical, chemical, and microbial fading (Wesenberg *et al.*, 2003). Although a some physical-chemical treatments have been applied for decolorization of wastewater, these processes present high operational costs and limited applicability as main disadvantages (Ali, 2010). Alternatives based on conventional biological processes are relatively ineffective for treating dye wastewater (Fu and Viraraghan, 2001).

This has resulted in considerable interest in use of biological systems for treatment of dyestuff. Recently, microbial decolorization, involving suitable aerobic/anaerobic bacteria or fungi, has attracted an increasing interest (McMullan *et al.*, 2001; Mohorcic *et al.*, 2006). However, the main restriction to anaerobic bacterial treatment of azo dye is that the amines formed are not metabolized further under anaerobic conditions (Stolz, 2001). Furthermore, anaerobic decolorization operations are affected by

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chemical structure of dyes (Fu *et al.*, 2002). In some cases, dyestuff cannot be used as sole carbon source; therefore, extra carbon source may be added into decolorization media (Bras *et al.*, 2001). There are some restrictions of bacterial aerobic dye degradation since it is confined to chemostat-enriched cultures to a single dye (Saratale *et al.*, 2011). Limitations and inefficiencies in current treatments have led researchers to consider different methods and microorganisms for efficient decolorization.

The most extensively studied dye-decolorization microorganisms are white rot fungi (Maximo and Costa-Ferreira, 2004; Kalpana *et al.*, 2012). Use of fungi and their oxidative enzymes is an alternative method for treatment under aerobic conditions (Yesilada *et al.*, 2002; Cing *et al.*, 2003; Revankar and Lele, 2007; Zhuo *et al.*, 2011). Decolorization can be provided by adsorption of dye to fungal mycelium or by oxidative degradation of dye molecule (Fu and Viraraghan, 2001; Park *et al.*, 2007a). Thus, white rot fungi can be effectively used as an alternative to other traditional decolorization processes (Ali, 2010).

White rot fungi produce different intra-extracellular enzymes involved in pollutant degradation (Blanguez *et al.*, 2004). Recently, numerous studies have demonstrated that laccase (benzenediol:oxygen oxidoreductase) may be suitable for treatment of waste water (Soares *et al.*, 2001; Zeng *et al.*, 2012). However, decolorization and laccase production can also be influenced by different cultivation conditions and chemical structure of dyes (Mayer and Staples, 2002). In previous studies, bioremediation activities of repeated-batch cultures were compared with batch cultures of white rot fungi and it was stated that use of pellets in repeated-batch process may offer some advantages over batch mode, such as reduced sensivity to dyes and adverse operating conditions (Yesilada *et al.*, 2002; Cing and Yesilada, 2004; Birhanli and Yesilada, 2006). Repeated-batch mode offers an easy and practical method for decolorization of dyes and dyestuff in a long-term operation. In the present study, immobilized cells were investigated for decreasing handicap of free pellets in repeated-batch process. Immobilization provides distinct stability over free cells (Rodriguez Couto, 2009). Environmental factors, fungal immobilization, and repeated-batch conditions have also been investigated. The main purpose of the present study was to use effectively same fungal pellets in repeated-batch decolorization operations, to increase the number of pellet usability without adding any extra nutritional source and to compare the decolorization ability of free and immobilized fungal pellets.

Materials and Methods

Dyes : C.I. Acid Blue 74 (Indigoid) and C.I. Reactive Blue 198 (Diazo) were used for the study.

Fungi : White rot fungi, *Funalia trogii* ATCC 200800 and *Trametes versicolor* ATCC 2008001 were kept at 4 ºC after sub culturing at 30 ºC every 2-3 week on slant Sabouraud dextrose agar (SDA).

Preparation of free fungal pellets : Fungi were cultured at 30 ºC on SDA slants. After 1 week, mycelial suspensions were prepared and used for cultivation of inoculums. Mycelial suspensions were transferred into 250 ml flasks with 100 ml Sabouraud dextrose broth. Fungal pellets were formed after 5 d under 30ºC and 150 rpm agitation and then homogenized under sterile conditions. The homogenized samples were inoculated into each flask containing 600 ml fresh SDB. Fungal strain was grown for second growth cultivation for 5 days in a shaking incubator at 30ºC. After incubation, free fungal pellets were harvested by filtration from culture media and wet pellets were used in decolorization for further experiments. Dye concentration of each dye was 50 mg l^{-1} in all decolorization experiments.

Preparation of immobilized fungal pellets : For immobilization studies, fungi were cultured and inoculums were cultivated as stated above. *T. versicolor* and *F. trogii* cultures were agitated on rotary shaker at 30 ºC and 150 rpm for 4-5 days and gently homogenized under sterile conditions. In order to prepare immobilized fungal cells on activated carbon, 2 ml homogenate was transferred into 100 ml SDB medium, which also contained 0.3 g activated carbon powder, in 250 ml flask. Dimensions of activated carbons were 0.710-1.18 mm. Flasks' cultures were incubated in a rotary shaker at 150 rpm and 30 °C for 5 days. Immobilized pellets were harvested and used for decolorization experiments.

T. versicolor and *F. trogii* were grown on 0.710-1.18 mm pinewood cubes as solid support. Before inoculum, 0.2 g cubes were autoclaved in 40 ml SDB in 250 ml flasks and then they were inoculated with 2 ml homogenate and incubated at 30 °C for 2-5 hr statically. After static incubation, cultures were incubated on a rotary shaker at 110 rpm and 30 ºC for one day. After fungi were colonized on solid support, immobilized cultures were incubated at 150 rpm and 30 ºC for 4-5 day and then the medium was drained and immobilized pellets were aseptically transferred to other flasks for decolorization experiments.

Free fungal pellets were blended with 1% (w/v) sodium alginate for 15 sec. The mixture was added drop-wise into a cold solution of 0.1 M $CaCl_2$ and then immobilized culture was transferred into normal saline solution. Before being used for decolorization tests, beads of immobilized cells were grown for one day in a shaken culture (150 rpm) at 30 ºC in SDB. After they grew, the amount of fungal mycelium in immobilized cell beads increased with a corresponding enlargement in the size of immobilized culture. Immobilized cell beads were then washed gently with sterile distilled water and further evaluated for their decolorization ability. In order to determine the adsorption of dyes on fungal cells entrapped in alginate beads, free alginate beads were prepared and incubated at same culture conditions. Thus, adsorbed dye concentration was determined and decolorization

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rates were calculated accordingly.

Repeated-batch experiments : Free and immobilized pellets were prepared as mentioned above. The cultures which contained 50 mg l^{-1} dye in 50 ml distilled water and 3 g wet free or immobilized pellets were incubated at 30 ºC and 150 rpm. The retention time was 24 hr during the all repeated-batch operations. Pellets were taken from the test cultures aseptically after retention time and added into fresh media. This process continued during repeated-batch experiments. The media contained only dye and distilled water. No other nutrient was added in media in repeated-batch operation. Fungus-free abiotic controls were prepared and incubated in similar conditions.

Assay : Degree of decolorization was measured spectrophotometrically by using UV/vis spectrophometer (UV-1601 Schimadzu). Abs_{max} (an absorbance at the maximum peak) was obtained as absorbance at maximum peak for each dye. Percentage decolorization was calculated from absorption values against control. Dry weight of pellets was obtained by filtering cultures through filter paper and drying them to constant weight at 65 ºC. Laccase activity was determined spectrophotometrically by monitoring the increase in absorbance at 420 nm. One unit was defined as the amount of enzyme that oxidized 1 µmol of ABTS (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) per minute (Soares *et al.*, 2001). All the values were mean of at least three replicates ±SD.

Gel electrophoresis and staining : Before electrophoresis, the crude laccase filtrate was concentrated with 15000 molecular weight cut-off (MWCO) membrane concentrators (Milipore centricon). In order to determine the molecular weight of partially concentrated enzyme, SDS-PAGE was performed on 10% resolving and 4% stacking gels. The protein bands were visualized by silver staining methods (Heukeshoven and Dernick, 1998) and compared with standard molecular weight markers (Sigma-M2789): α-lactalbumin, 14.2 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; albumin (BSA), 66 kDa; phosphorylase-B, 97.4 kDa; β-galactosidase, 116 kDa; myosin, 205 kDa.

Gel renaturation and activity staining : For the activity staining, SDS was removed by washing gel at room temperature in solutions containing 2.5%(v/v) Triton X-100 for 30 min and 1.21 g^{-1} Tris-base, 6.30 g l^{-1} Tris-HCl, 11.7 g l^{-1} NaCl, 0.74 gl^{-1} $CaCl_2$ and 0.02 % (v/v) Tween 80 for 30 min, respectively. Renaturation of enzyme proteins was carried out by placing the gel overnight in a solution containing 50 mM sodium acetate buffer (pH 5), 5mM β-mercaptoethanol and 1mM EDTA EDTA at 4 ºC. The gel was then transferred onto a glass plate, sealed with film, and incubated at 37 ºC for 4–5 hr and then washed with 100 mM sodium acetate buffer (pH 5) (Unyayar *et al.*, 2005a). Zymogram analysis for laccase activity was performed by using 0.3% (w/v) ABTS 100 mM sodium acetate buffer (pH 5.5) at 30 ºC.

Results and Discussion

Preliminary experiments in liquid media were performed in order to determine decolorization ability of free pellets (*F. trogii* and *T. versicolor*). Highest decolorization with agitation rates of 150 rpm was found at 30ºC. Similar and high decolorization rates were obtained with all the tested free pellet concentrations of both fungi in 24 hr, but decolorization efficiency of low concentration of free pellets was insufficient during the repeated-batch experiments (data not shown). The results showed that high pellet amount gave good decolorization yield during long-term operation. Optimal dry weight of free pellets of *T. versicolor* and *F. trogii* were 0.59 and 0.82 g 50 ml^{-1}, respectively. Media with initial pH values in the range of pH 3-10 supported good dye decolorization efficiency by these fungi (Table 1). The results showed that it is not necessary to adjust the initial pH of these types of dye effluents (Yesilada *et al.*, 2002) because instead of growing them at different pH, the whole pellets were directly used to decolorize the dyes and these pellets were not affected by initial pH changes. However, pH of the medium has an important role for pellet production (Borras *et al.*, 2008). This pH tolerance of decolorizing whole pellets is quite important since it makes them suitable for practical biotreatment of effluents containing dyes.

Each dye showed different decolorization tendency and indigoid-based dye decolorized faster than azo-based dye (Table 2). Acid Blue 74 was decolorized 96 and 94% within 2 hr by *T. versicolor* and *F. trogii*, respectively (Table 2). In the first four cycles of repeated-batch studies, decolorization ability of *T. versicolor* pellets against Acid Blue 74 was higher than *F. trogii* pellets (Fig. 1a, b) and the average decolorization efficiencies of *T. versicolor* and *F. trogii* were 92 and 87% for four cycles. Similarly, fungal pellets showed high decolorization ability against Reactive Blue 198. Reactive Blue 198 was decolorized 87 and 84% within 2 hr by *T. versicolor* and *F. trogii* (Table 2). *T. versicolor* free pellets displayed high decolorization ability both in terms of extent and rapidity of decolorization of Reactive Blue 198 (Fig. 2a). The average decolorization efficiencies of *T. versicolor* and *F. trogii* pellets were 88 and 62% for six cycles, respectively. In addition, *T. versicolor* decolorized these dyes without any visual dye sorption onto pellets. As shown in Fig. 3, free pellets of *T. versicolor* and *F. trogii* were colorless after the first cycle of repeated-batch decolorization. Laccase activity of *T. versicolor* free pellets, during repeated-batch decolorization of Reactive Blue 198, was lower as compared to *F. trogii* pellets in all the cycles (Fig. 4). These results are in accordance with the findings of Casas *et al.* (2013). They reported low laccase production with *T. versicolor* during repeated-batch mode, but high decolorization of Alizarin Red. According to Casas *et al.* (2013), low laccase production was due to lack of supplemented nitrogen source. In the present study, additional nutrients were not added to the medium. Blanquez *et al.* (2004) found close relationship to intracellular laccase and degradation of Grey Lanset G by

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Table 1 : Effect of initial pH on decolorization (%; after 24 hr incubation) of Acid Blue 74 (AB74) and Reactive Blue 198 (RB198) by free pellets of *F. trogii* (FPF) and *T. versicolor* (FPT) at 30 ºC and 150 rpm.

	Decolorization (%) of AB74 by		Decolorization (%) of RB198 by	
Initial pH	FPF	FPT	FPF	FPT
3	94.63±0.336	96.01±0.027	85.89±0.455	90.70±0.105
4	96.10±0.216	96.16±0.280	87.22±0.160	90.87±0.220
5	93.70±1.109	96.33±0.055	88.58±0.929	90.32±0.241
6	94.88±0.166	96.30±0.027	86.52±0.929	90.91±0.377
7	93.17±1.188	96.17±0.082	84.81±0.756	91.19±0.201
8	93.84±0.193	95.55±0.219	84.31±5.341	91.71±0.060
9	94.26±0.705	95.62±0.138	86.76±0.483	92.09±0.056
10	94.98±0.663	95.53±0.028	88.85±0.680	89.83±0.422

Values were expressed as mean±standard deviation of 3 analysis

Table 2 : Decolorization (%; after 2–24 hr incubation) of Acid Blue 74 (AB74) and Reactive Blue 198 (RB198) by free pellets of *F. trogii* (FPF) and *T. versicolor* (FPT) at 30°C and 150 rpm.

	Decolorization (%) of AB74 by		Decolorization (%) of RB198 by	
Incubation (h)	FPF	FPT	FPF	FPT
2	94.00 ± 0.19	96.35 ± 0.07	84.05 ± 0.31	87.43 ± 0.06
4	94.47 ± 0.09	96.48 ± 0.18	84.88 ± 0.22	88.02 ± 0.06
6	94.94 ± 0.33	96.62 ± 0.02	85.65 ± 0.12	88.16 ± 0.12
8	95.20 ± 0.22	96.63 ± 0.02	86.21 ± 0.28	89.20 ± 0.13
10	95.96 ± 0.09	96.63 ± 0.03	87.11 ± 0.16	90.42 ± 0.06
12	96.28 ± 0.15	96.75 ± 0.05	87.43 ± 0.16	90.80 ± 0.10
24	96.14 ± 0.02	96.84 ± 0.05	87.88 ± 0.10	91.36 ± 0.40

Values were expressed as mean± standard deviation of 3 analysis

Trametes versicolor. Not only extracellular but also intracellular laccase might play an important role in decolorization of Acid Blue 74 and Reactive Blue 198 by *Trametes versicolor*. Laccase activity of *F. trogii* was the highest on the first day; approximately 0.500 U ml^{-1}. After first cycle, enzyme activity and decolorization efficiency of fungal pellet were decreased. Decolorization mechanism of fungi is not so clear. Indeed, there are many factor that affect fungal decolorization. One of the most important factors is different chemical structure of dyes (Park *et al.*, 2007a). Swamy and Ramsay (1999) reported that complexity of structure was not only an indicator of difficulty of decolorization of azo dyes but breaking of azo bond also depended on the identity, number, and position of functional groups in the aromatic region. Dominguez *et al.* (2005) indicated that decolorization was only due to intra- and extracellular enzymes produced by fungi during the operation. Other important factor was physiological diversity of fungi that could cause difference in fungal decolorization activity (Park *et al.*, 2007b). Zhang and Yu (2000) indicated disadvantages in using of free pellets. When free pellets reach a certain relatively large diameter, diffusion of nutrients and oxygen into center of mycelium mass is too slow to maintain the unrestricted growth of entire mycelium. Thus, hyphae at the center may even die leaving an empty core (Zhang and Yu, 2000). Therefore, number of usage of pellets in repeated-batch operation decreased. In order to evaluate the effectiveness of the pellets (especially *F. trogii* pellets) and to increase the number of repeated usage of pellet in dye decolorization, immobilize cells were tried into or onto the support materials to compare decolorization ability of fungal pellets immobilized with different methods.

Longevity of decolorization with immobilized *T. versicolor* was measured during repeated-batch experiments. Fig. 1a illustrates that Acid Blue 74 decolorization performance and stability of free pellets and immobilized fungal cells on pinewood was similar but higher than other immobilization operations after fourth cycle. As compared to free pellets (83%), immobilized fungal cells on pinewood (87%) increased the average decolorization efficiency in first six cycles. Immobilized fungal cells on activated carbon and fungal cells entrapped in alginate beads retained their high decolorization performance for only four cycles. Average decolorization efficiency of both immobilized fungal cells was 96 and 92%, respectively, but after first four cycles decolorization ability of immobilized cultures decreased drastically. Alginate-immobilized *T. versicolor* decolorized Amaranth at the same rate in repeated-batch culture containing

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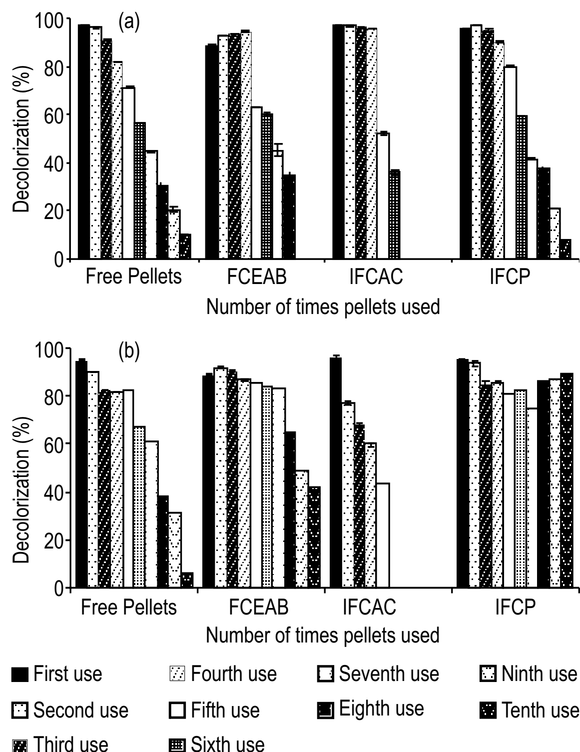

Fig. 1 : Decolorization of Acid Blue 74 dye by free and immobilized pellets of *T. versicolor* (a) and *F. trogii* (b) under repeated-batch operation at 30 °C and 150 rpm

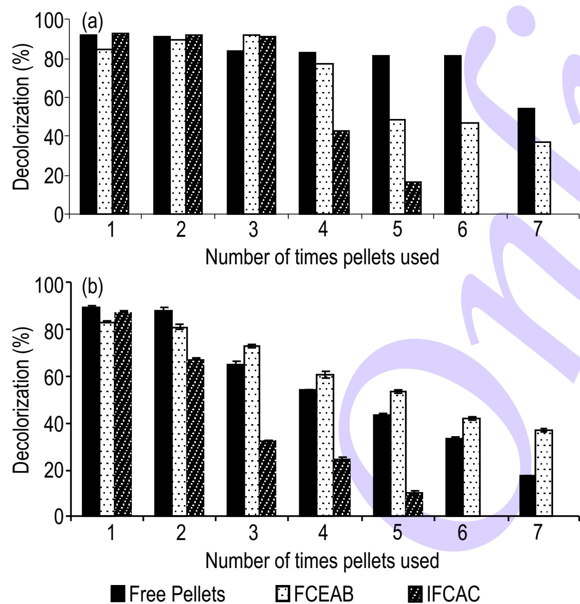

Fig. 2 : Decolorization of Reactive Blue 198 dye by free and immobilized pellets of *T. versicolor* (a) and *F. trogii* (b) under repeated-batch operation at 30 °C and 150 rpm

either ammonium tartrate or glucose alone (Ramsay *et al.*, 2005). But in the present study, no additional nitrogen and carbon source was added. The dye molecule and pinewood might be consumed as nitrogen and carbon source**.** Initially, the laccase activity of free pellets (0.36 U ml^{-1}) was higher than immobilized pellets but with re-using of pellets it decreased (0.11 U ml^{-1}). Libra *et al.* (2003) reported that the enzyme activity measured in the medium was not a reliable parameter for evaluating decolorization capacity because most of the active enzymes were retained in the fungal slime layer during immobilization. Reactive Blue 198 decolorization performance of FCEAB remained high and stable for at least 4 cycles and the average decolorization efficiency of free pellets and FCEAB was 88 and 85%, respectively (Fig. 2a). When *T. versicolor* cells were immobilized on activated carbon, decolorization rate of Reactive Blue 198 was high during the first three cycles but this immobilization technique was not effective on later reusability of pellets (Fig. 2a).

F. trogii immobilized on pinewood retained its decolorization activity against Acid Blue 74 longer than *T. versicolor* immobilized on pinewood (Fig. 1a, b). Therefore, this was the most effective immobilization method to obtain high Acid Blue 74 decolorization activity. The microenvironment might be changed in the presence of pinewood and become more favorable for dye decolorization. The results showed that immobilized pellets were still capable of Acid Blue 74 decolorization after 10 cycles. Decolorization percentage remained high (86%) and laccase activity also enhanced with availability of pinewood (Fig. 5). Dye was observed to be biosorbed to immobilized pellets during first hour of incubation and then dyed pellets became colorless. Therefore, a mechanism was proposed that included first step of adsorption of dye in the biomass, followed by the step that transferred it into cells and the dyes were decolorized enzymatically (Romero *et al.*, 2006; Yesilada *et al.*, 2010). *F. trogii* immobilized on pinewood was able to produce laccase and decolorize dye (Fig. 5) (Park *et al.*, 2007a). Similar results were reported by Mazmanci and Unyayar (2005) in decolorization of Reactive Black 5 by *Funalia trogii* immobilized on *Luffa cylindrica* sponge. Mazmanci and Unyayar (2005) found relationship between decolorization rate of Reactive Black 5 and laccase activity of *F. trogii* and reported that laccase enzyme was involved in decolorization. Entrapping fungal cells in alginate beads affected longevity of decolorization positively. The experiments were performed for 10 days and decolorization efficiency still remained higher than 83% at an early stage of repeated-batch experiments. High laccase activity (0.49 U ml^{-1}) decreased after the first use of immobilized pellets (Fig. 5).

During first cycle, Reactive Blue 198 decolorization ability of all the tested cultures was high (83-90%), but then decolorization ability of fungal cells, immobilized on activated carbon, decreased rapidly (Fig. 2b). The average decolorization efficiency, obtained after six reuse of fungal cells immobilized in alginate beads (66%), was higher than free pellets (62%).

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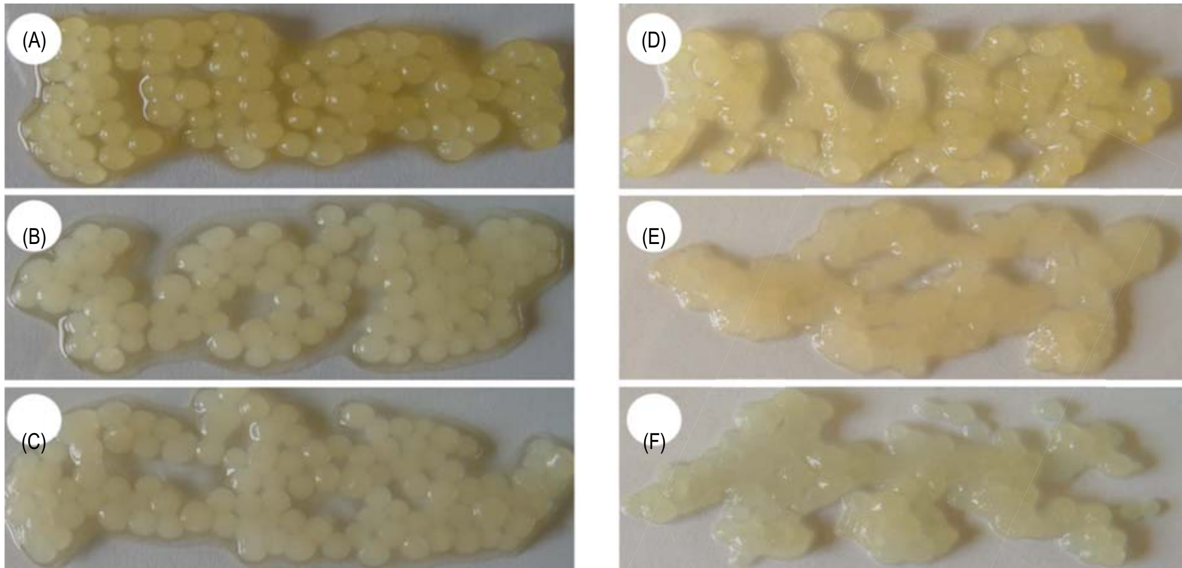

Fig. 3 : Photographs of *T. versicolor* and *F. trogii* pellets before and after dye decolorization. *T. versicolor* pellets before (A) and after Acid Blue 74 (B) and Reactive Blue 198 (C) decolorization. *F. trogii* pellets before (D) and after Acid Blue 74 (E) and Reactive Blue 198 (F) decolorization

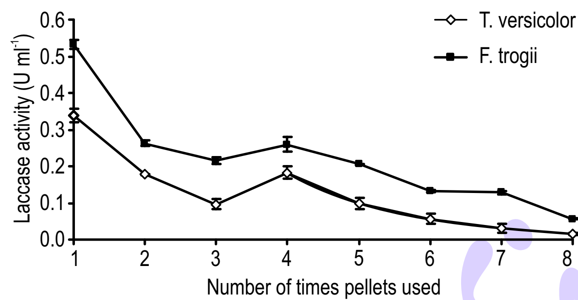

Fig. 4 : Laccase activity in the test solution of free pellets during repeated-batch decolorization of Reactive Blue 198

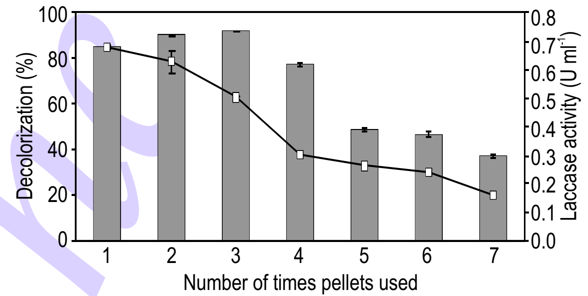

Fig. 6 : Laccase activity in the test solution of *F. trogii* immobilized pellets (FCEAB) during the repeated-batch decolorization of Reactive Blue 198

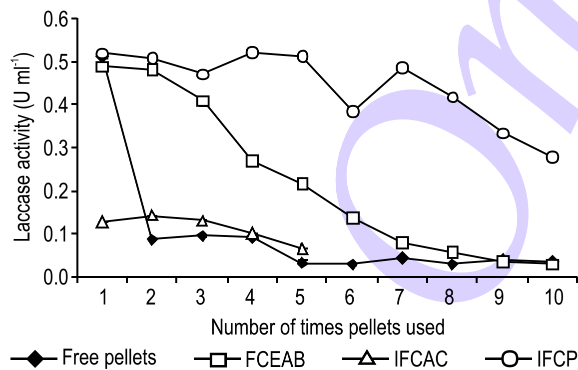

Fig. 5 : Laccase activity in the test solution of *F. trogii* free and immobilized pellets during the repeated-batch decolorization of Acid Blue 74

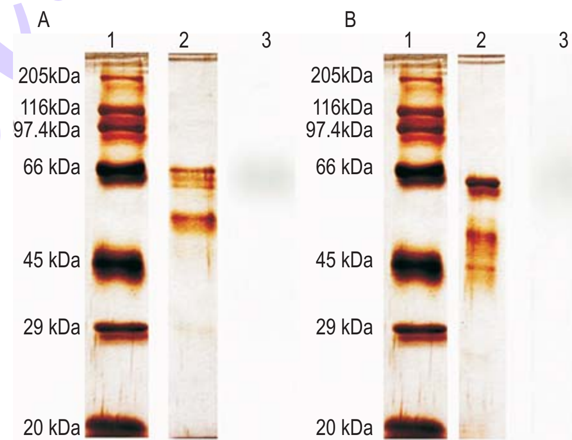

Fig. 7 : SDS-PAGE and zymogram of culture filtrate of *T. versicolor* (A) and *F. trogii* (B). Lane 1, molecular weight markers; Lane 2, culture filtrate; Lane 3, zymogram of renaturated laccase enzyme

Maximum laccase activity of FCEAB was 0.676 U ml^{-1}. It was obtained after first use and remained at high level for additional three uses and then decreased slightly (Fig. 6).

Crude laccase filtrates obtained from optimum conditions were concentrated and molecular weight of the enzyme was determined by SDS-PAGE with molecular weight marker. In order to determine the molecular weight enzyme band, activity staining was done with laccase substrate (ABTS) after gel was renatured. After sufficient staining (3-5 min), a single green colored zone appeared on the band for *T. versicolor* and *F. trogii*. The calculated molecular weight of bands was 64 kDa and 61 kDa (Fig. 7). Deveci *et al*. (2004) and Unyayar *et al*. (2005b) reported that a single band (65 kDa) was detected according to the results of SDS polyacrylamide gel electrophoresis of laccase activity of *F. trogii*. Lorenze *et al*. (2006) determined two laccase isoenzymes bands (60 and 65 kDa). These results are in accordance with the results of present study.

F. trogii pellets were softer than *T. versicolor* pellets. *F. trogii* pellets dispersed into mycelia during the operations. Therefore, immobilization could protect *F. trogii* pellets from shear damage. Thus, *F. trogii* pellets immobilized on pinewood could be used for 10 times during repeated-batch mode. Repeated use of pellets makes decolorization process economical. In addition, decolorization process could be achieved in hours, which increased the application of this method.

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