Journal of Hazardous Materials 169 (2009) 88–93

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Involvement of ligninolytic enzymes of *Phanerochaete chrysosporium* in treating the textile effluent containing Astrazon Red FBL in a packed-bed bioreactor

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ARTICLE INFO

*Article history:*
Received 10 September 2008
Received in revised form 14 December 2008
Accepted 17 March 2009
Available online 26 March 2009

*Keywords:*
COD
Decolorization
Kissiris
Peroxidase
*Phanerochaete chrysosporium*

ABSTRACT

The effect of Tween80, Mn(II) and veratryl alcohol (VA) on the production of ligninolytic enzymes of *Phanerochaete chrysosporium* in a packed-bed bioreactor using small pieces of Kissiris as carrier, was investigated. The results of the enzyme activities were noticeable in terms of decolorization and COD removal of the textile effluent containing an azo dye (Astrazon Red FBL). No dilution was made on the tested textile effluent and it was not sterilized, also. Maximum decolorization of the dye (87%) and COD removal (42%), both occurred when only Tween80 (0.05%, w/v) was added to the effluent. The maximum activities of lignin peroxidase (LiP) and manganese peroxidase (MnP) were (U/l): 17 and 52, respectively. The role of MnP was pronounced in the dye decolorization process, while the influence of LiP was noticeable on COD removal. The reusability of the original biomass was examined by replacing undiluted textile effluent (i.e., five times). The cellular performance of the original biomass in repeated-batch operations was promising.



## 1. Introduction

The ever-growing list of chemical contaminants released into the environment on a large scale has intensified search for selection a proper bioremediation strategy. The effluents of dyeing, printing, photographs, cosmetics, pharmaceutical and textile industries contain dye [1,2]. The estimates show that over $7 \times 10^7$ tons dyes are produced annually worldwide and 5–10% of these amounts of dyes are lost in industrial effluents [3,4]. On the basis of the chemical structure of the chromophoric group, dyes are classified into: azo dyes, anthraquinone dyes and phthalocyanine dyes. Azo dyes are commercially important group since these make up about 50% of the total amount of dyestuffs used [5]. Textile industry is the largest sector for utilization of the reactive azo dyes which are known to hydrolyze during application and this may end up to 30% of the dyes being released through the effluents [6]. In addition, the dyes released are highly stable in sunlight and are typically not eliminated from water by traditional biological wastewater treatments. Moreover, most known physical and/or chemical technologies do not achieve the goal of the proper decolorization of colored effluents since these methods either are encountered operational difficulties or are expensive to apply. Under anaerobic conditions the azo linkage in azo dyes can be reduced to form aromatic amines which are colorless but more toxic than the parent molecules [7].

The potential use of lignin-degrading white-rot fungus *Phanerochaete chrysosporium* for the treatment of textile industrial effluents has been reported [2,8–10]. The lignin-degrading white-rot fungus *P. chrysosporium* is very active in nature through releasing extracellular, oxidative type of enzymes. The fungus therefore, plays an important role in degradation of lignin and organopollutants. Manganese-dependent peroxidase (MnP) catalyzes the oxidation of Mn(II) to Mn(III) in a reaction requiring certain organic acid produced by the fungus and chelates manganese and the resulting Mn(III) complex diffuses away from the active site of the enzyme and acts as diffusible oxidant which in turn can oxidize a variety of phenolic compounds [11]. Lignin peroxidase (LiP) catalyzes oxidation of non-phenolic electron-rich aromatic rings to the corresponding radical cations [12]. Role of laccase in lignin degradation is well established in *Pycnoporus cinnabarinus* culture, a fungus that lacks MnP and LiP [13]. Based on the results reported recently, it is suggested that the *P. chrysosporium* genome does not encode a typical laccase but rather encodes a multicopper oxidase with strong ferroxidase activity [14].

Effectiveness of degradation of the dyes by *P. chrysosporium* is dependent on providing suitable conditions preferably similar to the natural living environments of the fungus. Controlled growth of mycelia structure of the fungus and consistent production of appropriate amount of ligninolytic enzymes for extended periods are all dependent on developing a bioreactor system. In fact, production

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doi:10.1016/j.jhazmat.2009.03.070

of the ligninolytic enzymes is sensitive to shear forces and different low-shear approaches were taken [15,16]. A variety of immobilization systems are used and nylon-web, polyurethane foam, porous plastic material, silicon tube and sintered glass are common immobilization supports reported for the production of the fungal peroxidases [17]. Some important factors in selecting an adequate cell support are porosity, rigidity, inertness, availability and cost. Kissiris is a mineral glass foam material with high porosity formed by the foam thickening of volcanic lava [18]. Immobilization of *Kluyveromyces marxianus* on Kissiris for the production of ethanol has been reported [18]. Use of Kissiris in immobilization of *P. chrysosporium* in decolorization of methylene blue was reported previously [19]. Production of ligninolytic enzymes in various bioreactors using immobilized *P. chrysosporium* has been reported [20,21].

Decolorization of Astrazon Red FBL by *Funalia trogii* pellets has been studied [22] In the present study the influence of Tween80, Mn(II) and VA as the selected substances on the LiP and MnP production by *P. chrysosporium* in a packed-bed bioreactor employing pieces of Kissiris as cell support was investigated. The fungus performance therefore, on decolorization and COD removal of the textile effluent containing an azo dye (Astrazon Red FBL) was studied. The reusability of the original biomass was examined by replacing the effluent.

## 2. Materials and methods

### 2.1. Microorganism and growth medium

*P. chrysosporium* (1557) was purchased from the Deutche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) and was maintained at low temperature 4 °C, on 2.5% malt extract agar slants. Subcultures were routinely made every 2 months. Inocula consisted of conidial suspensions and were diluted so that the absorbance at 650 nm was 0.5, in a 1 cm path-length cuvette. The number of spores was determined using a Thuma counting—cell under the optical microscope at ×1000 magnification ($2.4 \times 10^6$ cells $ml^{-1}$). For preparation of conidial suspension, spores were taken from 2-week-old slants. This suspension was used to inoculate (10%, v/v) the growth medium.

Nitrogen-limited synthetic growth medium, which was used in the present study, had following composition ($l^{-1}$ of distilled water): glucose 10 g; $KH_2PO_4$ 2 g; $MgSO_4{\cdot}7H_2O$ 0.5 g; $CaCl_2$ 0.1 g; $MnSO_4$ 0.03 g; NaCl 0.06 g; $FeSO_4{\cdot}7H_2O$ 6 mg; $CoCl_2$ 6 mg; $ZnSO_4{\cdot}7H_2O$ 6 mg; $CuSO_4$ 6 mg; $AlK(SO_4){\cdot}12H_2O$ 0.6 mg; $H_3BO_3$ 0.6 mg; $Na_2MoO_4{\cdot}2H_2O$ 0.6 mg; Nitrilotriacetate 0.09 g; yeast extract 0.012 g; di-ammonium tartarate ($C_4H_{12}N_2O_6$) 0.2 g; thiamin 1 mg; veratryl alcohol 0.07 g and Tween80 0.5 g. The pH of the medium was adjusted to 4.5 using 20 mM sodium acetate. The growth medium composition was based on the culture composition given elsewhere [23,24], but preliminary work in our laboratory showed that some components could be reduced or completely eliminated without any major adverse effect. The above-mentioned formulation was thus used in the present study. The prepared medium was autoclaved at 9 psi for 30 min.

### 2.2. Support preparation

The bioreactor was filled with small pieces (~1 cm diameter) of Kissiris (external surface area determined by BET method: 4.9642 $m^2\ g^{-1}$), which acted as a supporting matrix on which the mycelium was bound. The Kissiris pieces were pretreated by soaking in the NaOH solution (0.1 M) for a few minutes followed by soaking in the $H_2SO_4$ solution (0.05 M). The pieces were washed with distilled water several times until the pH of the solution was ~pH 6.5 and no longer acidic. The pieces were then placed in a beaker in a boiling water bath for 10 min followed by drying them in an oven at 105 °C overnight. The pieces were finally sterilized in an oven at about 180 °C for 1 h.

### 2.3. Bioreactor configuration and operating conditions

A fixed-bed tubular bioreactor, consisted of a glass column (inner diameter 5 cm; height of supporting matrix ~20 cm; working volume 0.32 l) filled with pieces of Kissiris, was employed to perform the present study. The bioreactor was maintained at 37 °C by standing at temperature-controlled water bath. The temperature of the bioreactor system (including the upper part of the column) did not fluctuate and any temperature range was negligible. Air was supplied by a laboratory air compressor in a continuous flow of ~0.5 vvm after sterilization by filter.

### 2.4. Culture conditions

Cultivation was carried out by adding the growth medium to the bioreactor. This medium was inoculated with 10% (v/v) conidial suspension. 24 h after inoculation, the first signs of fungal mycelia growth was seen and the support was completely colonized by fungal mycelia within 2 days. After 2 days of operation and immobilization of microorganisms on solid materials, the liquid from the reactor was discharged and it was re-filled with undiluted, non-sterile effluent. The effect of different additives (Tween80 0.05% (w/v); $MnSO_4$ 1, 35, 110 ppm and veratryl alcohol 0.2 mM) in ligninolytic enzymes production and subsequently color and COD removal was investigated in each batch. After 4 days, each batch was finished and the reactor was prepared for starting a new batch. Finally the repeated-batch tests were done by replacing the aqueous phase with a fresh effluent volume for a total of five changes.

### 2.5. Effluent characteristics

The azo dye containing effluent characteristics were as following: pH, 5; $\lambda_{max}$, 521 nm; COD, 1500–1600 ppm; BOD, 124 ppm; color, light red.

### 2.6. Decolorization determination

Decolorization was represented by a decrease in absorbency at 521 nm. Hence, dye decolorization is reported as the relative change in absorbance ($A_t/A_0$) of the effluent after a certain time of treatment ($A_t$) to the initial absorbance ($A_0$).

### 2.7. COD determination

The chemical oxygen demand (COD) was determined according to standard methods. COD was measured spectrophotometrically using the dichromate closed reflux assay with a HACH test kit (HACH, LO vibond, CO).

### 2.8. Mn(II)-dependent peroxidase activity assay

The manganese peroxidase (Mn peroxidase) activity was measured according to the method pointed out elsewhere [25]. Complex formation of Mn(II) ions with malonate can be monitored spectrophotometrically at 270 nm (extinction coefficient = 11,590 $M^{-1}\ cm^{-1}$). One unit (U) is the amount of enzyme that catalyses the formation of Mn(II)-malonate complex (μmol/min).

### 2.9. Lignin peroxidase activity assay

Lignin peroxidase activity was determined spectrophotometrically according to Tien and Kirk [23] ($\lambda$ = 310 nm, extinction coefficient = 9300 $M^{-1}$ $cm^{-1}$). One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of VA in 1 min, and the activities were reported as U/l.

## 3. Results and discussion

### 3.1. Effect of Tween80

Several authors have shown an improvement in enzyme excretion in the presence of certain surfactants such as Tween80 in immobilized and submerged cultures of fungi [26–28]. The effect of Tween80 on LiP activity during the production of the enzyme in submerged agitated cultures of *P. chrysosporium* has been studied and the suggestion was that the effect of the detergent has a physiological basis for instance, improvement of the cell membrane permeability [26].

As shown in Fig. 1, by adding Tween80, MnP values were significantly higher than those obtained in the control culture (without Tween80) and low LiP activities were also detected. The presence of higher amounts of MnP did not improve the decolorization levels obtained (only 10%), but the COD reduction was increased about 18% (Table 1). These observations show that lower values of MnP (obtained in the control culture) were sufficient for decolorization. It also indicates that the minimum MnP value necessary for COD removal is higher than that which is necessary for decolorization. Furthermore, the presence of LiP can be effective in COD reduction (this is more noticeable in investigating the effect of $MnSO_4$).

### 3.2. Effect of $MnSO_4$

Manganese is essential for the activity of MnP and it regulates the production of MnP and LiP in *P. chrysosporium* and in other white-rot fungi [11]. In lignin degradation process, work of Perez and Jeffries showed LiP is repressed as long as Mn is in solution as a chelator-stabilized equilibrium mixture of Mn(II)–Mn(III). Reappearance of LiP occurs once the mixture of Mn(II)–Mn(III) has precipitated as $MnO_2$ and removal of Mn (in the form of $MnO_2$ deposits) facilitates degradation of lignin by LiP [11].

Effect of Mn(II) at different concentrations on decolorization of sulfonphthalein dyes by *pleurotus ostreatus* has clearly shown the involvement of MnP in the process [29]. Decolorization of the anthraquinone-based Remazol Brilliant Blue R by *Irpex lacteus* in a packed-bed bioreactor was achieved at 100% within 10 days [30]. Polyurethane foam (PUF) and pine wood were used as the cell support for this white-rot fungus. Involvement of MnP in the decolorization process is reported and the activities were ranged between (U/l): 52 and 95 [30].

The effect of different concentrations of Mn(II) along with the constant level of Tween80 was tested. As shown in Fig. 2, the different manganese levels had no noticeable influence in MnP activity. LiP formation was inhibited completely in all concentrations of $MnSO_4$. Since $Mn^{2+}$ did not enhance MnP activity, it could be resulted that there was already sufficient $Mn^{2+}$ present to induce maximal MnP activity. MnP activity reduction found at high $Mn^{2+}$ concentration (i.e., $MnSO_4$: 110 ppm) was due to its repressive effect. It has been suggested that LiP synthesis begins only after soluble Mn is completely removed by precipitation of $Mn^{4+}$ as $MnO_2$. Not LiP production could be due to presence of $Mn^{2+}$.

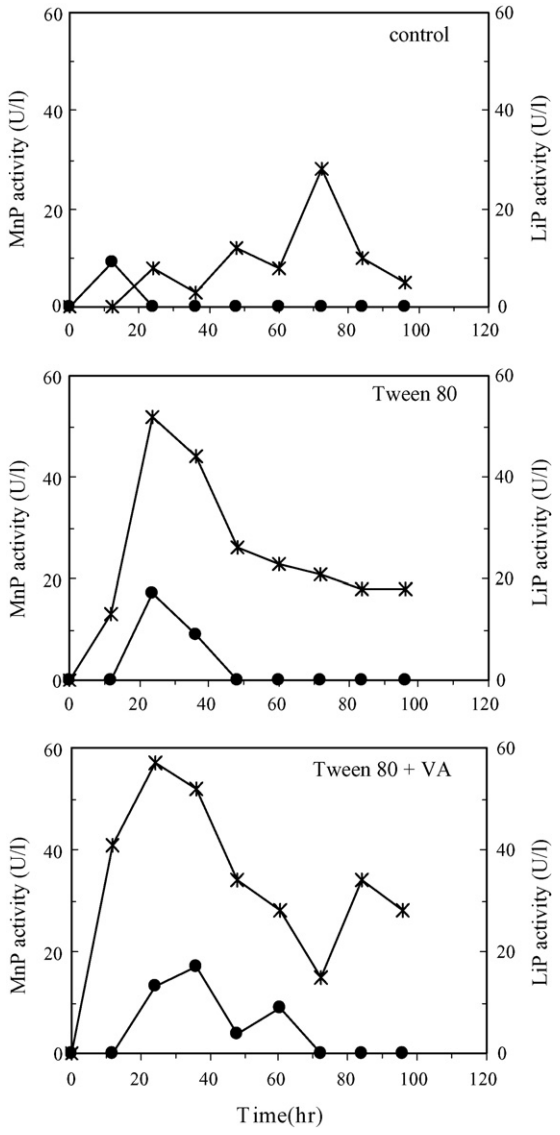


**Fig. 1.** Ligninolytic activity in cultures of *P. chrysosporium* immobilized on Kissiris in packed-bed bioreactor under different culture conditions. Symbols: ●, LiP activity (U/l); *, MnP activity (U/l).

The noticeable point is the same COD reduction in all $Mn^{2+}$ concentrations that is lower than the reduction obtained when only Tween80 was added (Table 1). The COD reduction was not

**Table 1**
Influence of Tween80, $Mn^{2+}$ and VA on decolorization, ligninolytic activity and COD reduction of the non-sterile effluent by *P. chrysosporium* immobilized on Kissiris in a packed-bed bioreactor.

| Decolorization conditions | COD reduction (%) | Decolorization (%) | Maximum LiP activity (U/l) | Maximum MnP activity (U/l) |
|---|---|---|---|---|
| Effluent alone | 25 | 78 | 9 | 28 |
| +Tween80 | 43 | 88 | 17 | 52 |
| +Tween80, $MnSO_4$ (1 ppm) | 33 | 88 | – | 50 |
| +Tween80, $MnSO_4$ (35 ppm) | 33 | 86 | – | 55 |
| +Tween80, $MnSO_4$ (110 ppm) | 33 | 80 | – | 38 |
| +Tween80, VA | 37 | 85 | 17 | 57 |

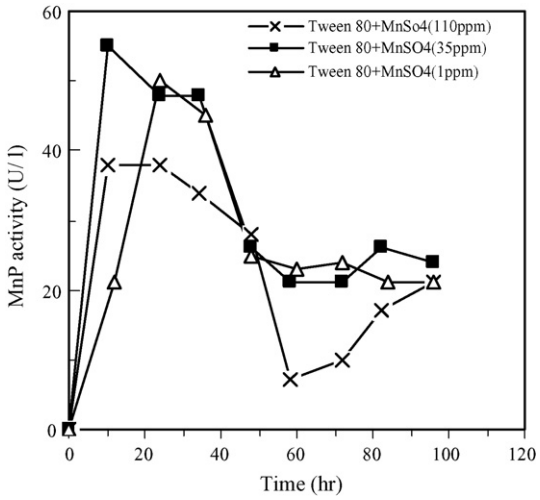


**Fig. 2.** MnP activity in cultures of *P. chrysosporium* immobilized on Kissiris in a packed-bed bioreactor under different concentrations of $MnSO_4$.

dependent on the concentration of the added manganese. COD reduction of the textile effluent may correlates to the decolorization of the particular compound, probably the azo dye. This also confirms the role of LiP in COD reduction. It is speculated that MnP performs the depolymerization of the highest-molecular-weight products. Subsequent reoxidation by MnP to Mn(II) results eventually in disproportionation to $MnO_2$. The LiP isoenzymes appear rapidly once the Mn(II)–Mn(III) has precipitated as $MnO_2$. This observation correlates with the onset in depolymerization to lower-molecular-weight product and more efficient degradation by LiP.

The results obtained also show that MnP has performed the depolymerization and caused the COD reduction, but because of not production of LiP, the COD reduction was lower than that obtained when only Tween80 was added. The decolorization capacity maintained in a high level except in the highest concentration of $MnSO_4$, which decreased a little as a result of lower MnP production (Table 1).

### 3.3. Effect of veratryl alcohol

Veratryl alcohol has a protective effect on LiP activity against inactivation by proteases [31]. Protective role of VA *in vitro* in the catalytic cycle of LiP during decolorization of polyaromatic azo dyes has been studied and it is suggested that the simultaneous oxidation

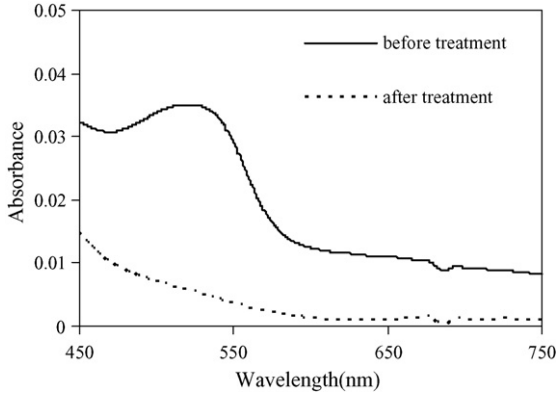


**Fig. 3.** Spectra of the undiluted, non-sterile dye effluent at different wavelengths before and after treatment with *P. chrysosporium* immobilized on Kissiris in a packed-bed bioreactor.

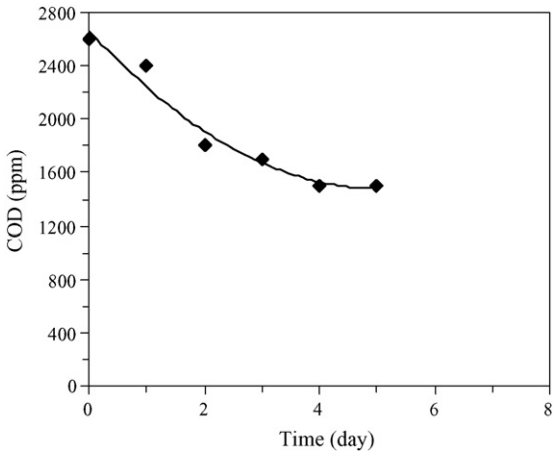


**Fig. 4.** The COD changes during the treatment of the non-sterile dye effluent by *P. chrysosporium* immobilized on Kissiris in a packed-bed bioreactor.

of VA with reduction of LiPII to the native state of LiP completes the oxidation of the azo dye [32].

According to our results, by supplementing the medium with VA, MnP activities did not change significantly. LiP activities were not higher noticeably but the presence time of LiP increased which could be due to protective effect of VA (Fig. 1). As the

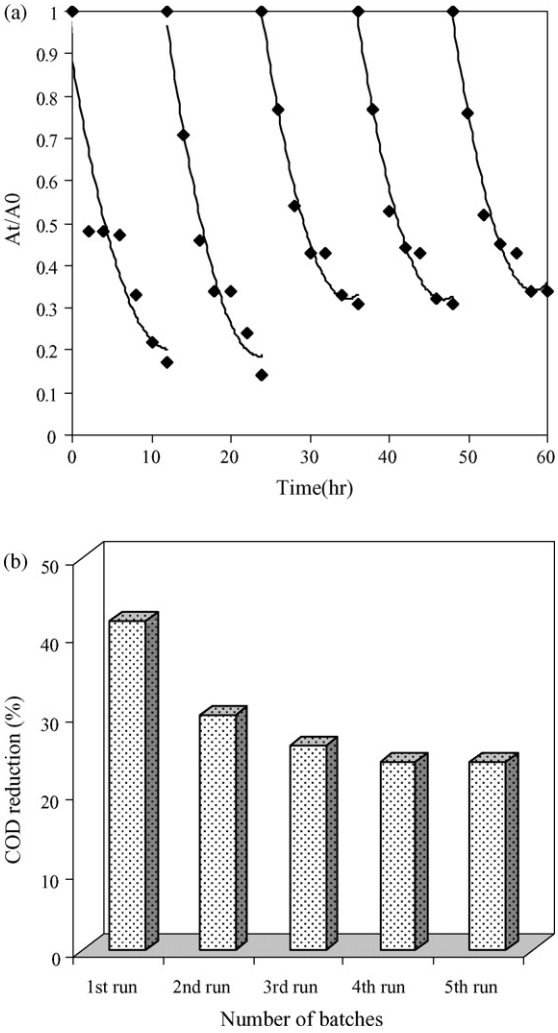


**Fig. 5.** Decolorization (a) and COD removal (b) of the undiluted non-sterile dye effluent by Kissiris-immobilized *P. chrysosporium* in a packed-bed bioreactor.

ligninolytic enzymes activities did not change significantly, the decolorization and the COD reduction did not change noticeably also (Table 1).

According to the results obtained and in order to prevent adding additives to the effluent as possible, the best results were obtained when just Tween80 was added. The spectra of the effluent before and after treatment are shown in Fig. 3. After decolorization, the absorbance at all wavelengths decreased with the largest decrease at the peak. A general decrease of this kind is usually attributed to dye degradation rather than adsorption. Fig. 4 shows the daily COD reduction. The COD reduction continued until the 4th day and after that it remained constant. This could be due to the limited ability of ligninolytic enzymes in cleavage of pollutants molecules.

### 3.4. Repeated-batch operations

Fig. 5 shows the results of repeated-batch operations. As the results show, after 5 sequential batches, the fungal capacity in decolorization is maintained in an acceptable level (decolorization results are shown in the first 12 h of each batch, the period that decolorization was almost complete). At the end of the fourth and fifth decolorizations, the final absorbance was higher than it should have been if based on color alone. This was due to an increase in turbidity as a result of the formation of microemulsion as it is reported elsewhere [9]. The fungus ability in COD reduction reached a constant level at the end of fourth and fifth batch (i.e., COD reduction: 26%). This shows the capability of the fungus in COD removal even a relatively small part.

## 4. Conclusions

Although numerous studies have shown that *P. chrysosporium* can decolorize a variety of dyes in defined media, this one demonstrates that it can decolorize and reduce the COD value of a dye effluent without adding any nutrients. It can be concluded that Kissiris is a suitable support for *P. chrysosporium* immobilization. Adding Tween80 (0.05%, w/v) as the best additive, increased decolorization up to 88% and COD reduction up to 43%. It appears that MnP has more pronounced effect on decolorization, while LiP is more effective in COD reduction. The repeated-batch tests demonstrate that immobilized *P. chrysosporium* can maintain high degrading activity for a long term operation. The fungal decolorization process could efficiently remove most of color from dye effluent (by breaking down the chromophoric molecules) and eliminated a relatively small part of COD from the effluent. Therefore a two-stage-treatment of decolorization, combined with another biological treatment like activated sludge, is proposed to solve the problem. Toxicity tests are not performed in the present work. Considering the toxicity is needed before use of the treated wastewater in the environment. So, the enzymatically treated wastewater should be evaluated for special applications such as irrigation, through conducting toxicity tests (i.e., phytotoxicity) as reported elsewhere [33,34].

## Acknowledgments

The authors sincerely thank Alireza Monazzami for his expert technical assistance as director of computer site.

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