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# Design of reaction conditions for the enhancement of microbial degradation of dyes in sequential cycles

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**Abstract**

The present study evaluated the potential of white-rot fungal strain *Coriolus versicolor* to decolorize five structurally different dyes in sequential batch reactors under optimized conditions. The experiments were run continuously for seven cycles of 8 d each. High decolorizing activity was observed even during the repeated reuse of the fungus, especially when the old medium was replaced with fresh medium after every cycle. Biodegradation was the dominating factor as the fungus was able to produce the enzyme laccase mainly, to mineralize synthetic dyes. The nutrients and composition of the medium played important roles in sustaining the decolorisation potential of the fungus. Corncob was found be an easy and cheap substitute for carbon source for the fungus. Glucose consumption by the fungus was in accordance to its decolorisation activity and chemical oxygen demand (COD) reduction.

**Key words**: *Coriolus versicolor*; decolorisation; sequential batch reactor

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## Introduction

Synthetic dyes are extensively used in a number of industries, such as textile dyeing and paper printing. These dyes include various types like acidic, basic, azo, reactive, anthroquinone-based compounds. The treatment of wastewater from textile and dyestuff industries is one of the most challenging tasks. The existing treatment technologies for dye removal suffer from several drawbacks such as high amounts of chemical usage and/or sludge generation, costly infrastructure requirements and/or high operating expenses (Nigam *et al*., 2000). The use of bacteria in the biological treatment of dye effluents may result in the generation of colorless, dead-end aromatic amines, which are generally more toxic than the parent compounds (Banat *et al*., 1996), therefore, may have poor adaptability and limited application to a wide range of dye wastewater (Kulla *et al*., 1983).

Fungal decolorisation processes offer a complete cleanup of pollutants in a natural way as it reduces the color components to carbon dioxide, ammonia and water by initiating cleavage of the bonds in dyes rather than creating possible toxic fragments of dyes. Basidiomycetous fungi are able not only to decolorize but also to degrade and mineralize a broad spectrum of different dye structures (azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes), in addition to numerous of other toxic organic and recalcitrant compounds. The enzymatic system involved in the degradation of pollutants by these fungi is nonspecific and even acts on mixtures of pollutants (Machado *et al*., 2005; Wesenberg *et al*., 2003). Wood rotting fungi have interesting properties in the sense. They are capable to degrade lignin which is a polymeric structure with a lot of aromatic rings. The fungi have been shown to excrete certain enzymes that catalyze the formation of activated oxygen, and then the process is initiated. White-rot fungi have been used in studies concerning decolorisation of different textile dyes (Knapp and Newby, 1999; Rodriguez *et al*., 2000; Mielgo *et al*., 2003). The lignolytic enzymes of the white-rot fungi are thought to be expressed in most cases during secondary metabolism when the carbon or nitrogen source becomes limiting, indicating a need of separate carbon source. Strains such as *Phanerochaete chrysosporium* and *Trametes*/(*Coriolus*) *versicolor* (Kirk and Farrel, 1987; Royer *et al*., 1985), have been found to be quite effective in decolorization of various kraft and pulp mill effluents (Archibald *et al*., 1990; Olfat *et al*., 2000; Mehra *et al*., 1995) and textile dyestuffs (Kapdan and Kargi, 2002; Heinfling *et al*., 1997).

We have previously studied the effect of parameters such as media composition, nitrogen source and age of fungus on the decolorisation of a representative azo RBV dye in batch reactor (Sanghi *et al*., 2005). The objective of the present study was to evaluate the potential

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of *Coriolus versicolor* (live cells) to degrade a broad spectrum of reactive synthetic dyes sequentially and to estimate the longetivity and sustainability of the fungus under toxic conditions created by the repeated spiking of dye. Enzymatic activity was monitored and accordingly the possible mechanism of decolorisation was discussed. For the design of continuous reactors for decolorisation, a cheaper alternate to otherwise expensive carbon source was also provided.

## 1 Materials and methods

### 1.1 Reagents

Stock solutions (100 mg in 1 L of tap water, Table 1) of different class of anionic, reactive, acid and direct dyes were prepared without further purification. Reactive Remazol Brilliant Violet (RBV), Reactive Procion Brilliant Blue RS (PBB), Direct Bordeaux Red (DBR), Acid Orange 7 (AO7), Acid Yellow (AY) dyes (Table 2) were purchased from Colour-Chem Ltd., Mumbai, India. Unless otherwise stated, except for AY (50 mg/L) the concentration used for all dyes was 100 mg/L which is much higher than the actual dye concentrations found in real textile wastewater.

### 1.2 Culturing of the fungus

*Coriolus versicolor* was obtained from Institute of Microbial Technology, Chandigarh, India. The strain was maintained at 4°C on malt agar slants. Erlenmeyer flasks of the capacity 150 mL was used for growing the fungal mycelia. It contained 50 mL of the growth medium, consisting of 5 g/L malt extract powder and 10 g/L glucose. The media was always autoclaved and cooled before use. The initial pH was 5.6 and all cultures maintained at 30°C.

**Table 1** Physiological properties of tap water

| Parameters | Permissible limit | Maximum limit | Tap water |
|---|---|---|---|
| pH | 6.5–8.5 | No relaxation | 8.15 |
| TDS (mg/L) | 500 | 2000 | 577 |
| Hardness (as $CaCO_3$) (mg/L) | 300 | 600 | 156 |
| Alkalinity (as $CaCO_3^{2-}$) (mg/L) | 200 | 600 | 470 |
| Nitrate (mg/L) | 50 | No relaxation | 1.09 |
| Sulfate (mg/L) | 200 | 400 | 58 |
| Fluoride (mg/L) | 1 | 1.5 | 1.2 |
| Chloride (mg/L) | 250 | 1000 | 60 |
| Turbidity (NTU) | 5 | 10 | 0.3 |
| Cadmium (mg/L) | 0.01 | No relaxation | BDL |
| Chromium (mg/L) | 0.05 | No relaxation | BDL |
| Lead (mg/L) | 0.05 | No relaxation | BDL |
| Iron (mg/L) | 0.3 | 1 | 0.19 |
| Zinc (mg/L) | 5 | 15 | 0.04 |

TDS: total dissolved solids; BDL: below detection level.

### 1.3 Batch kinetic experiments

Batch decolorisation experiments were carried out in 500 mL flasks. Each flask contained 400 mL of autoclaved culture medium comprising glucose 10 g/L, malt extract 5 g/L and dye was added to the cultures as aliquots of concentrated stock solutions. All the flasks except the control were inoculated with fungal pellets of the same size grown in the liquid culture.

All the experiments were carried out at 25°C. The reactors were continuously aerated by aquarium pumps at a rate of 1.02 L/min for 10 h and were static for the next 14 h. Autoclaved controls were set up with autoclaved carriers in all experiments to study abiotic dye removal. Four microlitters of the sample were withdrawn from each flask daily, centrifuged and analyzed for absorbance, COD and pH.

Studies were also conducted to evaluate effects of using alternate C-sources (5 g/L), like corn-cob, starch and lactose on the decolorisation of 100 mg/L RBV. Corn-cob was collected and dried at room temp for 2 d. It was cut into small pieces of approximately 2 cm diameter and 0.2 cm width. At a time one piece was used in a batch reactor. Blank experiments were also conducted with corn-cob as well as with dyes without using fungal pellets.

The unadjusted pH of the culture media was 5.4–5.6. As a general observation in almost all cases, there has been a decrease in the pH of the reactors as the decolorisation proceeds, it goes down to 3–3.5 and sometimes to 2.8, depending on the dye used. The decrease in initial pH did not have any effect on decolorisation (Knapp *et al*., 1995). As observed previously (Dirk *et al*., 2003; Ana *et al*., 2002) the fungus produces organic acids such as malonate, oxalate during the initial growth period could decomposed by the enzyme (manganese peroxidase). The fungus was biologically active during this period.

#### 1.3.1 Analytical procedure

The concentrations of different dyes were measured at a wavelength corresponding to their maximum absorbance, $\lambda_{max}$, by means of a UV-Vis spectrophotometer (UV-160A, Shimadzu, Japan). A standard curve was obtained for each dye with a concentration range 10–150 mg/L. The decolorisation was monitored by the percentage of reduction in absorbance at $\lambda_{max}$ in comparison with abiotic controls. The COD measurements were performed according to the standard of APHA (1992) using a HACH COD digestor, (USA). The pH of the solution was measured with a digital pH meter (MK VI, Systronics, India).

**Table 2** Details of the dyes used

| | C.I. index | Type | λ (nm) | Absorbence (max.) |
|---|---|---|---|---|
| Remazol Brilliant Violet (RBV) | Reactive Violet 5R, C.I. no 18097 | Vinyl sulphone | 560, 325 | 0.75, 0.62 |
| Procion Brilliant Blue (PBB) | Reactive Blue 4, C.I. no. 61205 | Dichlorotriazine | 632.49 | 0.95 |
| Direct Bordeaux Red (DBR) | Direct Red 28, C.I. no. 22120 | Diazoamino naphthalene sulphonate | 498.7 | 0.68 |
| Acid Orange 7 (AO) | Tropeolin OOO, C.I. no. 15510 | Naphthyl azo benzene sulphonic | 484, 308, 221.5 | 1.090, 0.465, 1.609 |
| Acid Yellow-2R (AY) max. | Acid Yellow 36, C.I. no.13065 | Anilino phenyl azo benzene sulphonic | 435, 255, 210 | 1.524, 0.865, 1.566 |

### 1.3.2 Enzyme assays

The lignin peroxidase activity was determined using the method described by Tien and Kirk (1988). Laccase activity was monitored using the method described by Leonowicz and Grzywnowicz (1981) and the manganese-dependent peroxidase activity was measured using the method described by Kuwahara *et al*. (1984).

## 2 Results and discussion

### 2.1 Adsorption/degradation

In addition to biodegradation, adsorption also plays an important role in the decolorisation mechanism of dyes by living fungi. For the adsorption process, it is not clear whether external binding only or external binding plus internalization take place (Bonnarme and Jeffries, 1990).

In order to evaluate the phenomenon by which decolorisation was taking place, some studies were initially conducted with 100 mg/L RBV as the representative dye. Three reactors (B1, B2 and R) containing the fungus in 100 mg/L dye solution were setup. B1 and B2 were designated as the blank reactors. B1 containing the culture media was autoclaved along with the fungus so as to kill the fungus whereby no biological activity associated with it. The live fungus in B2 thrived in distill water which was devoid of any nutrients in the form of culture medium.

The extent of color removal by adsorption alone in B1 was always limited (less than 20%), whereas in B2 in the first three days 26% color reduction was observed which increased to around 39% by the end of day 7 (Fig. 1). In the reactor flask (R), the combined decolorisation due to biodegradation and adsorption was 97% within 7 d. The decolorisation was accompanied by complete disappearance of the peak at 560 nm and was always associated with primary degradation. It was also observed that some of the dye remained adsorbed to the mycelium pellet during the incubation period. In an attempt to solubilise any bound dye, repeated extraction of the dye bound fungus, in alkaline medium (0.1 mol/L NaOH) was done over a course of 8 h with the help of ultrasonicator until all intact dye was released into the medium as evident by the fungus becoming whitish again. The resulting supernatant was combined and the degree of adsorption was then determined using the scanning profile.

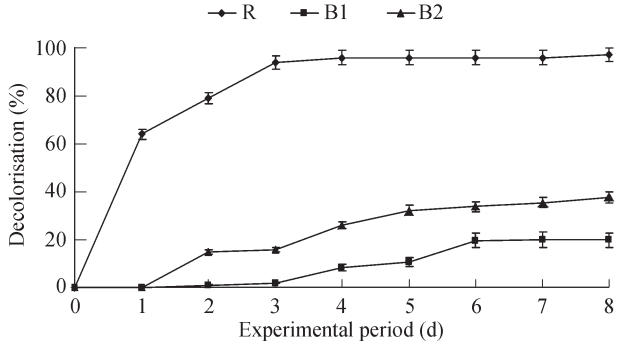


**Fig. 1** Adsorption vs. degradation by the fungus under different control conditions reactors (R, B1, B2).

The color of the fungal mycelia during the course of study was indicative of the fungal mode for decolorizing the dye solution. In the reactor R the fungal biomass attained a pinkish tinge on approaching day 7 and hardly any color (5 mg/L) could be extracted from the mycelium, indicating that the major dye removal is by biodegradation. In B1 it was dark violet on day 2 and most of it was extractable (18 mg/L of the dye out of 20 mg/L removed), indicating the phenomenon to be adsorption only. In B2 it was red-violet on day 3 and only 11 mg/L dye could be extracted from the fungus out of 39 mg/L dye removed during the process. The results clearly demonstrated that under enriched conditions the fungus was able to biodegrade the dyes whereas under nutrient deficient conditions, the mode of color removal by fungus was bio-adsorption as was evident by the color of the biomass in different reactors.

A separate blank experiment with corn-cob in 100 mg/L dye solution was also performed to determine the effect of corn-cob as an adsorbent. The corn-cob could adsorb around 5 mg/L dye within 3 d. For convenience this marginal amount was ignored in corn-cob studies.

### 2.2 Effect of initial dye concentration

Initial dye concentration is an important factor in determining the rate and extent of decolorisation in the experiments performed with RBV dye. For dye concentrations of 20, 50, 100 mg/L the rate and extent of decolorisation were similar and almost complete decolorisation within 2 d (Fig. 2). As expected, the decolorisation rate was very slow for higher dye concentrations and the maximum decolorisation could not be attained even after 8 d. Clearly, the high concentrations of dyes create toxic conditions for the fungus which makes it difficult to thrive and grow.

### 2.3 Effect of carbon source

Since fungi need at least one C-source to sustain, dye decolorisation is largely dependant on the type of carbon source used. Studies were conducted to evaluate the effect of using alternate C-sources (5 g/L), like corn-cob, starch and lactose on the decolorisation of 100 mg/L RBV. It was observed that although in all cases the decolorisation percentage on the last day was almost the same, but the rates varied a lot. The maximum decolorisation that could be achieved on day 8 was 95% with glucose, 94% with corn-cob, 94% with lactose and 45% with starch. The rate

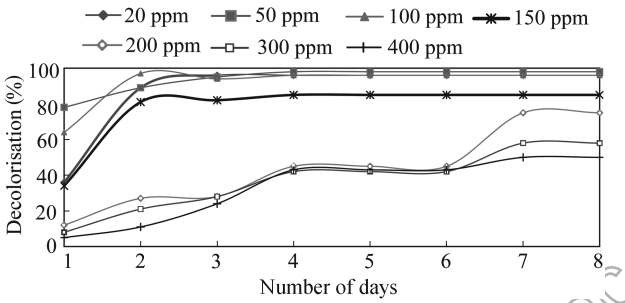


**Fig. 2** Effect of initial concentration of Remazol Brilliant Violet dye on decolorisation.

of decolorisation was almost similar for glucose and corn-cob, but varied widely for lactose and starch. On day 3, the decolorisation was only 16% and 25% for starch and lactose as compared to 66% and 80% for corn-cob and glucose, respectively. These findings suggest that corn-cob can be a vital and cheaper C-source substitute for the decolorisation.

### 2.4 Enzyme activity

Studies were performed to monitor the change in enzymatic activity during the process of decolorisation of RBV dye. Initially, the reactors were run with fungal biomass in the nutrient medium for two days, and then the RBV dye was spiked. Minimal activity was observed (0.05–1.5 U/mL), when there was no dye in the first two days. This may be attributed to the extracellular enzymes already present in the fungus, during the growth phase. After two days when the dye was spiked, an increase in the enzyme activity was observed which reached its maximum at day 3 and thereafter started declining so as to be zero by day 8 (Fig. 3). From the studies it is clear that although laccase is the major enzyme responsible for decolorisation, the participation of other enzymes (LiP and MnP) cannot be ruled out. The results clearly indicate that the decolorization by *C. versicolor* involves complex mechanisms such as a combination of biodegradation by extracellular enzymes and adsorption by cells. Biodegradation being the dominating factor as the fungus was able to produce the enzymes to mineralize synthetic dyes. However, the relative contributions of lignin, laccase and MnP to the decolorization of dyes may be different for each fungal strain and each dye.

### 2.5 Sequential decolorisation of different dyes

The reactors were set up for the sequential batch studies, using 100 mg/L RBV, PBB, DBR, AY and 50 mg/L AO7 dyes so as to investigate the decolorisation capacity of the white-rot fungi *C. versicolor*. Fresh dye was spiked at the beginning of each cycle and old media was replaced by fresh media at every cycle from 3rd cycle onwards. The biomass, in pellet form, was retained in the bioreactor throughout the experiment.

As shown in Fig. 4, the maximum decolorisation of 96%, 97%, 82%, 66% and 66% for RBV, PBB, DBR, AY and AO7 dyes respectively was attained on day 4 of the experimental setup. In case of AO7, 90% decolorisation was observed after ten days of the first cycle, while in AY it did not increase any further and remained just 66% till the completion of the first cycle. The percentage decolorisation in the 2nd cycle was comparable to that obtained in the 1st cycle for RBV, PBB, and DBR, and was found to be 89%, 94%, and 87% respectively on day 4 of the cycle. In the second cycle, decolorisation observed in AO7 and AY was only 32% and 36%, respectively on day 4 which increased upto 83% in case of AY, but no further change was observed for AO7, although in the first cycle AO7 showed a decolorisation of 90%. In the case of RBV, the color removal was 97% till the end of 4th cycle which gradually reduced to 47% by the end of 6th cycle. The fungus showed an excellent capacity to decolorize PBB and about 91% color removal was obtained till the end of the 6th cycle. For DBR, an average 80% decolorisation was obtained till the end of 6th cycle. AY showed an unusual pattern of decolorisation, by the end of 2nd cycle, and 83% color was removed but then in this case, not much decolorisation percentage or decolorisation rate could be attained. Similarly, for AO7, it can be said that it is toxic for the fungus (90% decolorisation), the average decolorisation of the dye was reduced to 28%–40% till 5th cycle and there after no decolorisation for the next two cycles. Decolorisation efficiency for all the dyes was significantly reduced after the 6th cycle. By the end of 7th cycle, the decolorisation obtained was 9%, 60%, 23%, 46% and 0 for RBV, PBB, DBR, AY and AO7, respectively. Although the fungal pellets could be used several times during the long term operation, for PBB and DBR the media (R) used was able to sustain the fungal activity for almost six cycles, whereas for RBV it could well sustain for four cycles and for AO7 and AY it did not go well beyond the 2nd cycle.

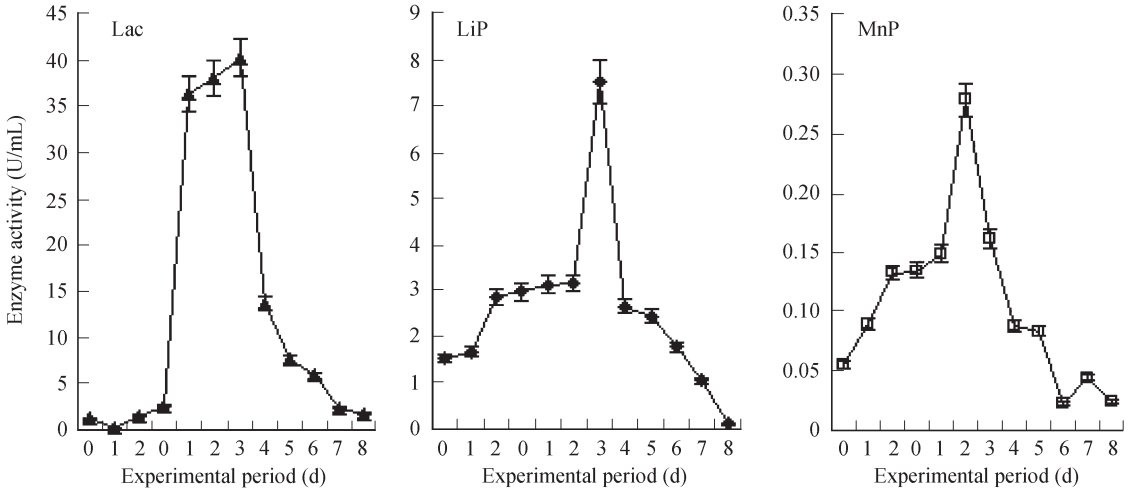


**Fig. 3** Enzyme activity.

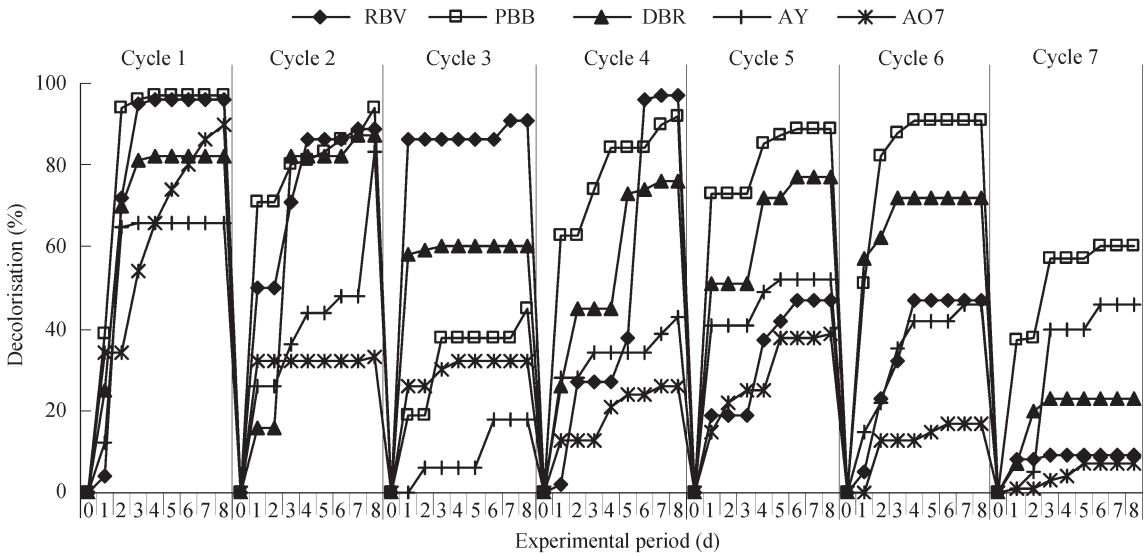


**Fig. 4** Sequential decolorisation of the dyes over seven cycles.

The spectral changes indicated that the decolorisation was caused due to the degradation of dyes (Mielgo *et al*., 2003). Even though the same amount of the inoculums was used for all the tested dyes, the differences found in the decolorisation characteristics for the individual dyes are attributed to the dissimilarity in specificities and structures of different dyes. Textile dyes vary greatly in their chemistries, and therefore their interactions with microorganisms depend on the chemistry of a particular dye and the specific chemistry of the microbial biomass (Polman and Brekenridge, 1996). In this case, acid dyes (AO7, AY) with benzenesulphonic nuclei were difficult to be degraded and are toxic to fungus in the initial cycles only. Fungus could sustain high decolorizing ability for the other dyes (RBV, PBB, DBR) fairly well till almost six cycles. Although higher concentrations of dye were found toxic to the fungus, it seems that the fungus can slowly adapt to such toxic levels over a course of sequential cycles in due course of time.

These results showed that the repeated-batch operations could decolorize the dye solutions at a much faster rate in subsequent additions, maintaining high decolorization activity for most of the dyes. It could thus reduce the toxic effect of high concentration of dyes. The eventual cessation of decolorization towards the later cycles is likely to be due to nutrient depletion. The differences that arise in the amount of decolorisation for different dyes are due to their different chemical structures.

The change in the COD was monitored daily during the course of decolorisation from first to 7th cycle. The extent of reduction in the COD values is almost similar (55% ± 10%) in all the cases irrespective of the dyes. It is well known that the removal of color is due to the cleavage of chromophore bond in the dye molecule. But the oxidation of aromatic ring compounds takes long time and hence the removal of COD may be less. Also, in sequential cycles the glucose concentration used (10 g/L) is more than what the fungus could consume. Therefore, the residual COD that remains could be partly attributed to the unused glucose and to fungal autolysis due to the fungus reaching its threshold level of dye toxicity.

## 3 Conclusions

*Coriolus versicolor* has been shown to efficiently decolorize five structurally different dyes during repeated-batch experiments under optimized conditions. The results clearly demonstrated that under enriched conditions the fungus was able to biodegrade the dyes whereas under nutrient deficient conditions, the mode of color removal by fungus was bio-adsorption. Even in the absence of additional supplement of nutrients in culture medium, the fungus could sustain its decolorizing potential well beyond six sequential continuous cycles. Although the general pattern of decolorisation for all the dyes followed a similar trend, it is clear that the fungi-dye interactions are dependant on the structure and functionality of the dye molecule.

It would also be of importance to evaluate the possibility to decolorize real textile wastewater containing different kinds of dyes with the aid of white-rot fungus in a process without the addition of glucose. Even though more studies on real wastewater are needed in the direction of substituting the medium constituents like glucose by easily available cheaper sources, corn-cob could indeed be a cheap substitute for glucose.

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