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Decolorization of Orange II Dye With the Crude Culture Filtrate of White rot Fungus, *Coriolus versicolor*

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Abstract: Decolorization of Orange II dye was demonstrated with crude culture filtrate of white rot fungus, *Coriolus versicolor*. It was determined that the rate and extent of decolorization were dependent on the age (growth phase) of the crude culture filtrate (CCF). Dye decolorization abilities of the CCF of *Funalia trogii, Pleurotus sajor-caju* and *Phanerochaete chrysosporium ME446*, after incubation for 12 days, were also studied. *Phanerochaete chrysosporium ME446* was observed to be unable to decolorize any of 4 dyes used. Cyanide and azide, which are known inhibitors of enzyme, inhibited dye decolorization ability of the CCF. Heat treatment terminated decolorization activity of CCF. The decolorization activity determined in this study was manganase independent activity and veratryl alcohol had no influence on it. Results showed that both H_2O_2 dependent and independent enzymes could play a role in the reduction of the color of Orange II dye. Moreover, this is the first study which correlates the age of culture filtrate with decolorization activity.

Key Words: Orange II, Dye, Decolorization, White rot fungi,

Coriolus versicolor Ham Kültür Filtratı Kullanarak Orange II Boyasının Renginin Giderimi

Özet: Bu çalışmada beyaz çürükçül fungus olan *Coriolus versicolor*'un kültür filtratının Orange II boyasının renk giderimi üzerine etkisi araştırılmıştır. Renk giderim hızı ve genişliğinin fungus ham kültür süzüntüsünün yaşına (üreme safhasına) bağlı olarak değiştiği saptanmıştır. 12 gün üretilmiş *Funalia trogii, Pleurotus sajor-caju* and *Phanerochaete chrysosporium* ME446'nın ham kültür filtratlarının da renk giderim yetenekleri araştırılmıştır. *Phanerochaete chrysosporium ME446* kullanılan 4 boyanın hiçbirinin rengini gideremediği saptanmıştır. Enzim inhibitörü olan siyanid ve azid, kültür filtratının boya rengini giderim yeteneğini inhibe etmişlerdir. Kültür filtratının ısı ile muamelesi de renk giderim aktivitesini sonlandırmıştır Bu çalışmada elde edilen renk giderim aktivitesi mangan bağımsız bir aktivitedir ve veratril alkolün etkisi yoktur. Sonuçlar Orange II nin renginin gideriminde hem H_2O_2-bağımlı ve hem de H_2O_2- bağımsız enzimlerin rol oynayabileceğini göstermiştir. Ayrıca, bu çalışma kültür süzüntüsünün yaşı ile renk giderim aktivitesinin ilişkisini ortaya koyan ilk çalışmadır.

Anahtar Sözcükler: Orange II, Boya, Renk giderimi, Bayaz çürükçül funguslar

Introduction

Dyes are commonly used in the textile, food and cosmetic industries and they are released into the environment in industrial effluents such as textile and dyestuff industries (1). Dyes used in this study are: 1) azo dye (Orange II), 2) triphenyl methane dye (Bromphenol Blue), 3) heterocyclic dye (Methylene Blue) and 4) starting material for polymeric dyes (Remazol Brillant Blue R). Various heterocyclic, azo and triphenyl methane dyes used widely by, e.g., the textile and dyestuff industry, are often resistant to biological wastewater treatment, and thus they are released into aqueous environment (2).

The microbial degradation and decolorization of dyes have received considerable attention from the viewpoint of treating industrial wastewater containing dyes. Azo dyes are the largest class of dyes (1). They are not readily degraded by microorganisms. Microorganisms that are able to degrade azo dyes anaerobically, have been isolated. However aromatic amines produced by all these anaerobic microorganisms may be toxic and carcinogenic (3). Wastewater treatment facilities are often unable to completely remove commercial dyestuffs, thus contributing to the pollution of aqueous habitats (4) . Some triphenylmethane dyes have been shown to be carcinogenic (4).

The lignin degrading white rot fungi mineralize a wide variety of structurally diverse environmental pollutants (5-8). Due to high oxidative potential of many of the enzymes associated with white rot fungi, e.g., ligninase, laccase, Mn-peroxidase, they have shown to exert a positive effect upon many potential environmental pollutants (9, 10).

There is considerable number of studies on decolorization and degradation of dyes by white rot fungi especially *P. chrysosporium* (1, 2, 11, 12). H_2O_2 dependent dye decolorization with concentrated culture filtrates has also been reported (13,14). So far, decolorization of dyes by culture filtrate has been investigated only in *P. chrysosporium*. There is not much information about the effects of the culture filtrate of white rot fungi on decolorization of dyes. Also there is almost no study about the effect of culture filtrates taken of different time intervals on the decolorization of the dyes.

The aim of this study is to test the decolorization ability of the CCF of the fungi, *C. versicolor* being the most studied one and to determine the effect of culture filtrates taken of different time intervals on the decolorization of the dye.

Materials and Methods

Fungi and Growth

In this study, white rot fungi *Coriolus versicolor*, *Funalia trogii, Pleurotus sajor-caju* and *Phanerochaete chrysosporium ME 446* were used. *C. versicolor* was identified by Işıloğlu and Watling (15) and *F. trogii* by Işıloğlu and Öder (16).

P. sajor-caju and P. chrysosporium Me 446 were received from N. Kolankaya, Microbiology Laboratory, University of Hacettepe. They were maintained by subculturing on sabouroud dextrose agar (Oxoid) at +4 °C every 2 to 4 weeks.

Dyes Used for Experiments

Bromphenol Blue (Merck), Methylene Blue (Merck), Orange II (Aldrich) and Remazol Brillant Blue R (Sigma) were used.

Decolorization Experiments with Whole Cultures of *C. versicolor*

C. versicolor was cultured at 28 °C in tubes on sabouroud dextrose agar. After one week of incubation, conidial suspension was prepared and used as inoculum. One ml of the suspension was transferred into a 20 ml liquid medium in a 250 ml flask. Culture was incubated at 30 °C without agitation. This medium contains (g/L): KH_2PO_4 0.2; $CaCl_2.2H_2O$ 0.1; $MgSO_4.7H_2O$ 0.05; $NH_4H_2PO_4$ 0.5; $FeSO_4.7H_2O$ 0.035; Glukoz 2; Yeast extract 1. All of the media used in this study were sterilized at 120 °C. After the growth of *C. versicolor* cells for 6 days, 200 µl of Orange II dye (1 mg/ml in water) was added. Orange II clearance from the culture fluid was monitored by assaying at A_{488}.

Decolorization Experiments with the CCF of *C. versicolor*

In order to test the effect of day of growth of the CCF on decolorization of the dyes, *C. versicolor* cells were allowed to grow for 20 days and culture filtrates were collected at 3-day intervals up to 20 days. They were filtered first with Whatman no:1 filter paper and then Millipore filter (0.45 µM pore size). *F. trogii, P. sajor-caju* and *P. chrysosporium* were culture for 12 days as indicated above. At this time the extracellular fluid was performed in a similar manner. These filtrates were used as crude culture filtrate for decolorization studies. The compounds were quantified by using the standard curves of absorbances versus concentration.

In order to test the influence of Mn11 (as $MnSO_4$), veratryl alcohol, H_2O_2, NaN_3, and KCN, they were added to the reaction mixture to given a final concentrations of 100 µM, 2 mM, 100 µM, 1 mM and 1mM respectively.

Heat treatment (80 °C for 30 min) and catalase enzyme (16-160 unit/ml) were used to determine whether decolorization activity was enzymatic or not.

To test whether the decrease in absobance was due to nonbiological oxidation or not, dyes were incubated with 100 µM H_2O_2.

Assays

Ten mL of each CCF was mixed with dyes and incubated at 30 °C for *C. versicolor*, *F. trogii* and *P. sajor-caju* and 39 °C for *P. chrysosporium*. The absorbance was measured at 591, 488, 662 and 590 nm to determine the concentration of Bromphenol Blue, Orange II, Methylene Blue and Remazol Brillant Blue R.

To determine whether the decolorization was a function of pH change, the effect of pH on the visible absorption of dyes was assayed by pH meter at different H^+ concentrations.

The dry weight of fungal mass was obtained by filtering the contents of each flask through preweighed Whatman no:1 filter paper and drying it to a constant weight at 70 °C. Yields were expressed as g of biomass per 200 ml of culture.

Laccase (O_2: p-diphenol oxido-reductase E.C.1.10.3.2) activity was determined by the oxidation of guaiacol and absorbance was read at 465 nm. The enzyme activity has been expressed in relative terms as colorimetric units (CU/mL) (17-19).

Results are the mean of three replicates.

Results

Decolorization of Orange II with Whole Culture of *C. versicolor*. The fungus was able to decolorize Orange II. The residual Orange II, was % 73 and % 46 of that initial concentration at 24 and 48 hours respectively. No initial absorbance chance was observed for the steril control cultures used. In order to see whether initial glucose concentration had an effect on the decolorization, two different concentrations (0.2 and 2 g/L) of glucose were used. There is no effect of glucose concentration an decolorization of Orange II by *C. versicolor.*

Table 1. Decolorization of Orange II by cultures of C. versicolor

Time (h)	Residual dye (µg/ml)	Culture pH
0	10±0.58	4.52±0.06
24	7.30±1.01	4.18±0.14
48	4.60±1.31	4.08±0.09
72	3.68±1.23	4.93±0.10

* Values are given as means± standard deviation

Decolorization of Orange II with the CCF of *C. versicolor*

C. versicolor which is a lignin degrader was used and decolorization ability of the filtered extracellular fluid (crude culture filtrate) was tested. Table 2 shows the growth, pH, enzyme activity and soluble protein change of *C. versicolor* during the growth in liquid media. The growth was obtained by sampling each flask containing 200 ml of medium and the whole fungal cell mass as described in materials and methods. The growth of fungus leveled off in 6th day and thereafter a slight decline in the dry weight was observed. The pH of the medium was also change with the growth of fungus in the culture medium. As shown in Figure 1 decolorization of Orange II with the crude culture filtrate was parallel to the growth of the fungus *C. versicolor* and extensive decolorization occured when the crude culture filtrate used was in secondary metabolic stage. An increase in the decolorization activity was determined after the trophophase of the fungus.

Figures 2, 3, 4, and 5 present the changes in the decolorization rate of CCF of *C. versicolor* when the initial concentration of Orange II dye was varied. The decolorization rate was increased with the age of the culture filtrate.

Table 2. Growth, pH, laccase activity and soluble protein changes of *C. versicolor* during growth in liquid medium

Days	Dry Weight (g/200 ml)	pH	Laccase Activity (CU/ml)	Soluble protein (mg/ml)
0	0.00	4.50±0.05	0.00	1.0±0.06
3	0.09±0.008	4.67±0.09	0.17±0.02	1.03±0.14
6	0.21±0.003	4.06±0.05	0.30±0.02	0.64±0.02
9	0.20±0.007	4.77±0.15	0.38±0.07	0.56±0.06
12	0.19±0.007	5.34±0.13	0.60±0.05	0.52±0.01
15	0.16±0.009	5.87±0.22	0.67±0.15	0.46±0.03

*Values are given as means ± standard deviation

The effect of starch (as a carbon source) on decolorization activity was also tested. For this, *C. versicolor* was incubated for 12 days. Then, the CCF was obtained by filtration and incubated with the Orange II as described in materials and methods. The decolorization rate slightly decreased by starch (Figure 6).

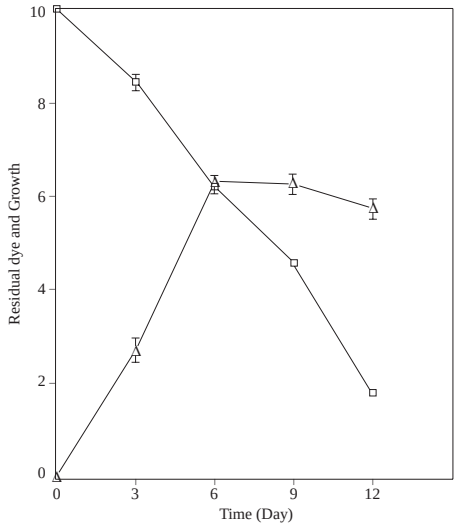

Figure 1. Decolorization of Orange II during the growth of *C. versicolor*. (Initial conc. 10 µg/ml). (Δ) Growth (g/200 ml x30) (❐) Residual dye after 24 hours (µg/ml)

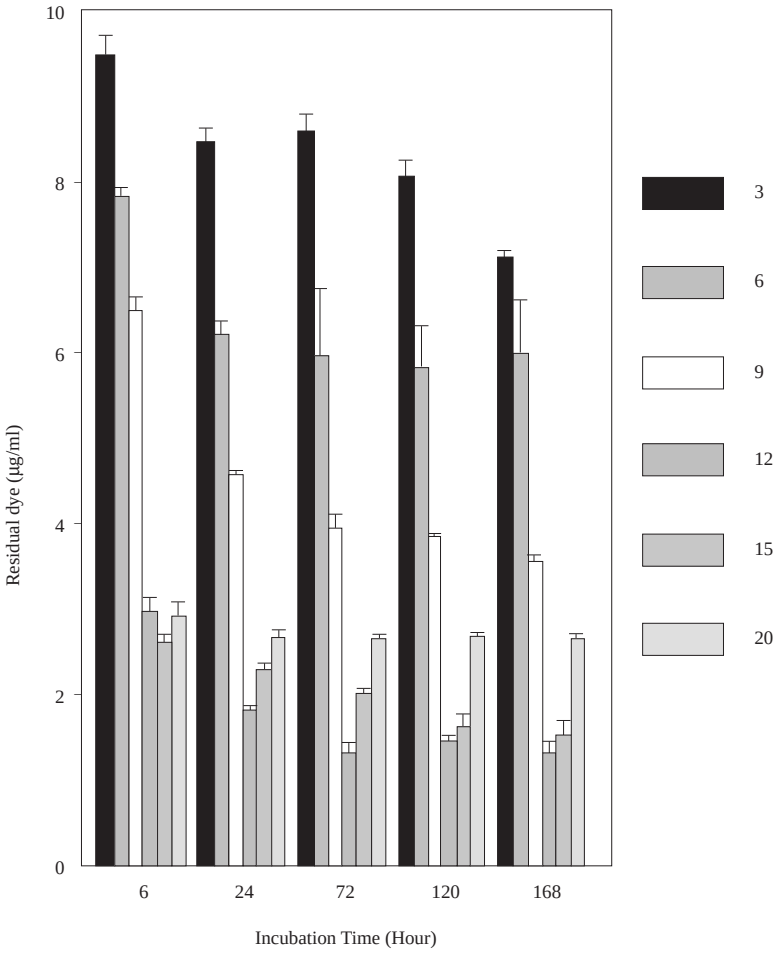

Figure 2. Effect of the age of crude culture filtrate of *C. versicolor on* decolorization of Orange II. (Initial conc. µg/ml) Error bars shown the standard deviation of the mean.

(3) 3 days old crude culture filtrate (12) 12 days old crude culture filtrate
(6) 6 days old crude culture filtrate (15) 15 days old crude culture filtrate
(9) 9 days old crude culture filtrate (20) 20days old crude culture filtrate

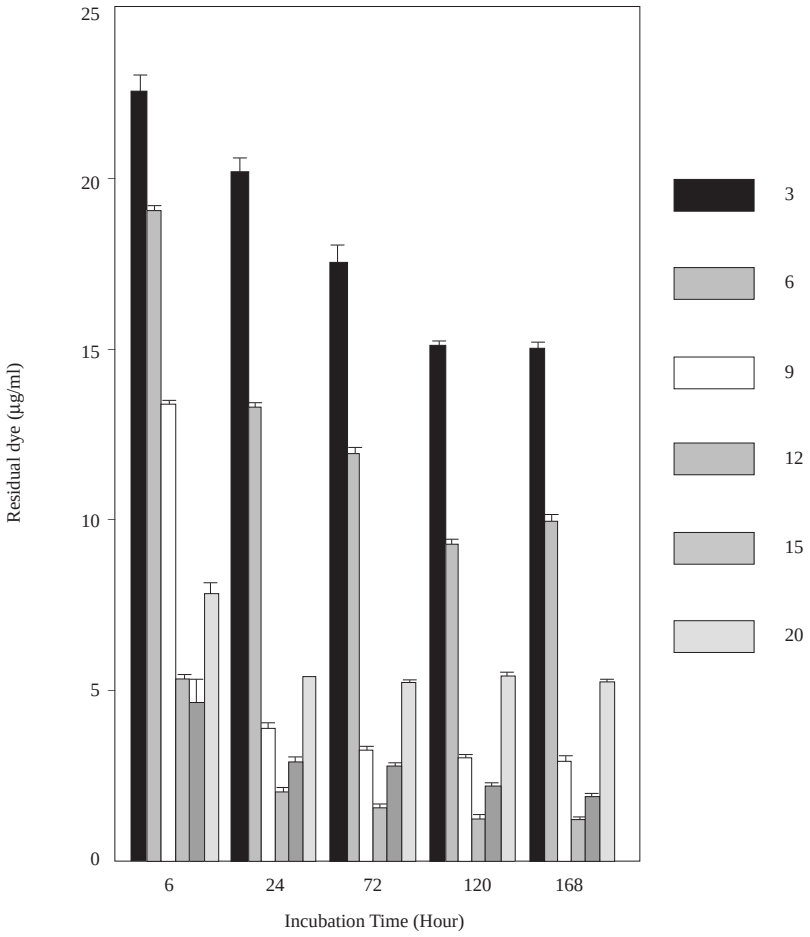

Figure 3. Effect of the age of crude culture filtrate of *C. versicolor* on decolorization of Orange II. (Initial conc. 25µg/ml).

Error bars show the standard deviation of the mean.

(3) 3 days old crude culture filtrate
(6) 6 days old crude culture filtrate
(9) 9 days old crude culture filtrate
(12) 12 days old crude culture filtrate
(15) 15 days old crude culture filtrate
(20) 20 days old crude culture filtrate

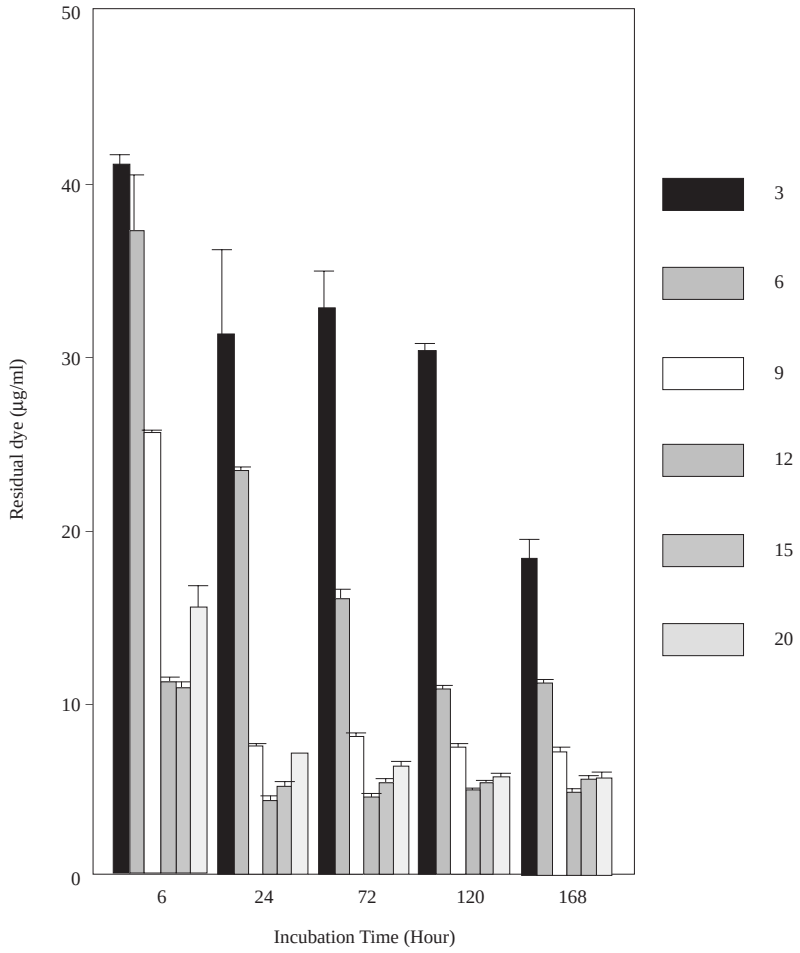

Figure 4. Effect of the age of crude culture filtrate of *C. versicolor* on decolorization of Orange II. (Initial conc. 50μg/ml).

Error bars show the standard deviation of the mean.

(3) 3 days old crude culture filtrate
(6) 6 days old crude culture filtrate
(9) 9 days old crude culture filtrate
(12) 12 days old crude culture filtrate
(15) 15 days old crude culture filtrate
(20) 20 days old crude culture filtrate

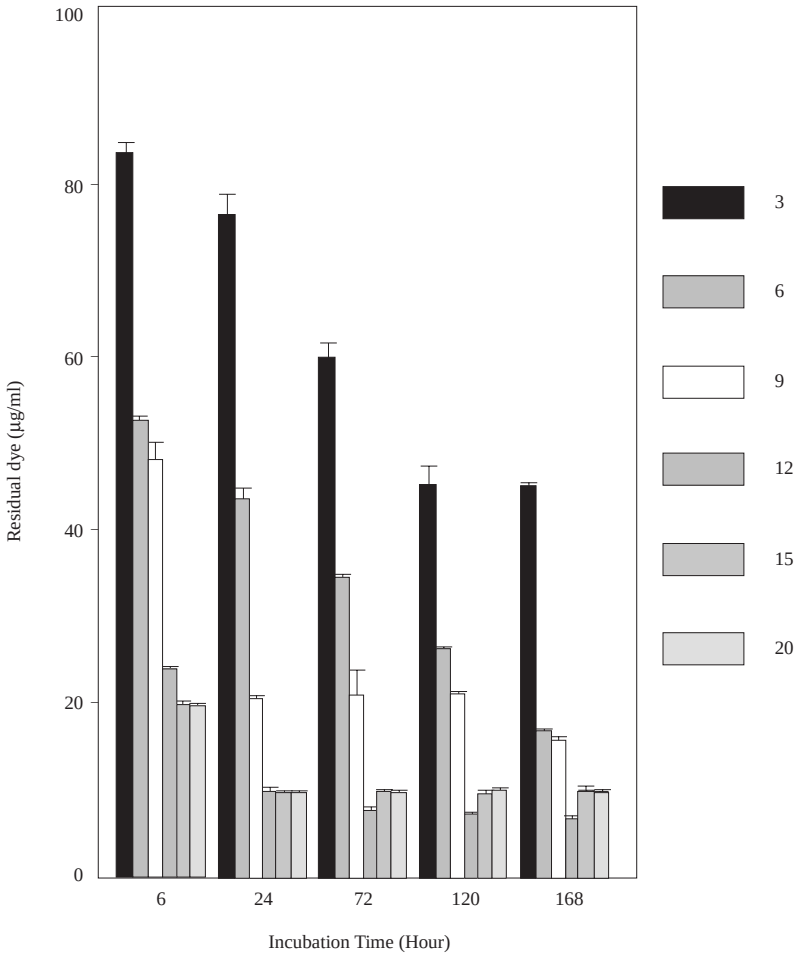

Figure 5. Effect of the age of crude culture filtrate of *C. versicolor* on decolorization of Orange II. (Initial conc. 100µg/ml).

Error bars show the standard deviation of the mean.

(3) 3 days old crude culture filtrate
(6) 6 days old crude culture filtrate
(9) 9 days old crude culture filtrate
(12) 12 days old crude culture filtrate
(15) 15 days old crude culture filtrate
(20) 20 days old crude culture filtrate

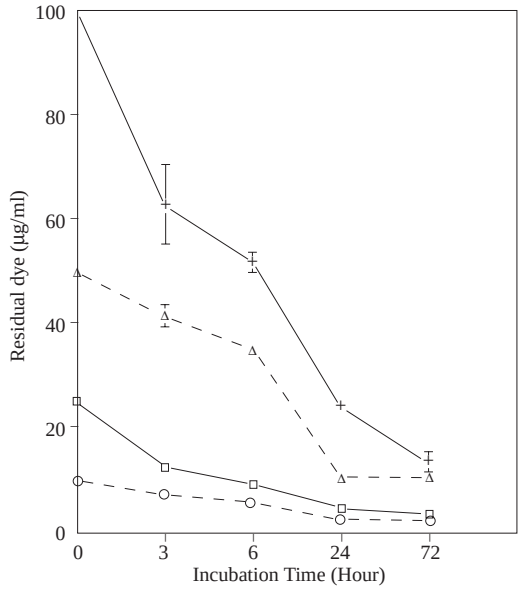

Figure 6. Effect of starch on decolorization of Oragne II.

Error bars show the standard deviation of the mean.

(+) 100 µg/ml Orange II (❒) 25 µg/ml Orange II

(Δ) 50 µg/ml Orange II (O) 10 µg/ml Orange II

Decolorization of Various Dyes with the CCF of *C. versicolor*, *F. trogii*, *P. sajor-caju* and *P. chrysosporium*

C. versicolor, *F. trogii*, *P. sajor-caju* and *P. chrysosporium* were incubated for 12 days and the effect of their crude culture filtrates on decolorization of Orange II, Remazol Brillant Blue R, Bromphenol Blue and Methylene Blue were tested. Table 3 shows the decolorization rate by these fungi after incubation with the dyes for 24 hours. CCF of *C. versicolor* shows higher decolorization rate for Orange II than that of the other three fungi. *F. trogii* also showed high decolorizing ability for Orange II and Remazol Brillant Blue *R. P. chrysosporium* had little or no activity on all the dyes used. Neither the crude culture filtrates of the fungi could decolorize Methylene Blue dye.

Influence of Mn^{11} (as $MnSO_4$) on decolorization ability of CCF

Decolorization of Orange II occured in the absence of exogeneously added Mn^{11}.

The decolorization ability of CCF was not enhanced by 100 µM Mn^{11}.

Table 3. Decolorization (%) of various dyes by the CCF of *C. versicolor, F. trogii, P. Sajor-caju* and *P. chrysosporium.*

Fungi	Bp.B.	Oran.II	RBBR	M.B.	Cul. Filt. pH
C. versicolor	70	92	81	0	5.97
F. trogii	32	89	87	0	6.07
P. sajor-caju	62	19	81	0	5.15
P. chrysosporium	9	9	0	0	5.56

* In this experiment 10 µg/ml Bp. B., 25 µg/ml Orange II., 100 µg/ml RBBR, and 20 µg/ml M. B.were used.

* Values are the mean of three replicate cultures.

* Bp. B: Bromphenol Blue; Oran. II: Orange II; R. B: B.: Remazol Brillant Blue R; M. B.: Methylene Blue; Cul. Filt. pH: Culture Filtrate pH.

Influence of veratryl alcohol and H_2O_2 on decolorization ability of CCF

The decolorization ability of CCF was not stimulated by exogenously added veratryl alcohol or H_2O_2.

Influence of NaN_3 and KCN on decolorization ability of CCF

Azide and cyanide are effective inhibitors of enzyme activity. At 1mM concentration, azide and cyanide were strong inhibitors. The decolorization ability of CCF for Orange II was inhibited by azide and cyanide both at 1 mM final concentrations. The effects of azide and cyanide on the dye decolorization ability of CCF are further evidence that this reaction is catalyzed by enzymes.

Influence of heat treatment on decolorization ability of CCF

The effect of heat treatment on dye decolorization ability of CCF was determined. Heating the CCF for 30 min inhibited the dye decolorization ability of this filtrate for Orange II. This shows that decolorization is an enzymatic event.

Influence of catalase enzyme on decolorization ability of CCF

To test that this activity was H_2O_2 dependent or not, CCF was first incubated with catalase enzyme (16-1600 unit/ml) and then dye decolorization ability of this filtrate was tested. When reaction mixture of Orange II was treated with catalase at 160 unit/ml, 86% Orange II decolorizing activity remained. But when catalase at 1600 unit/ml was used, 62% decolorizing activity remained. These results indicate that both H_2O_2 dependent and independent enzymes could play a role in the reduction of the color of Orange II dye.

During the study, it has been observed that the pH of the culture was continously changed. For this reason, the effect of pH on the visible absorption was assayed between pH 5 and 8 in the absence of CCF. The absorbance of the dyes were slightly effected by pH over this range (Table 4). This shown that decolorization can not be due to a pH change.

Table 4. The effect of pH on Decolorization (%) of the Dyes.

pH	Bromphenol Blue	R.B.B.R	Orange II
5.0	18	17	12
6.0	15	10	11
7.0	0	0	0
8.0	5	0	0

* Values are the mean of three replicates.

* Incubation Time : 24 hours

To exclude the possibility that the decolorization of the dyes was due to nonbiological oxidation, the dyes were incubated with 100 µM H_2O_2 in the absence of CCF. None of the dyes showed any change in absorption after incubation with H_2O_2.

Discussion

In this study decolorization of various dyes, especially Orange II, by the culture filtrates of white rot fungi was tested. Throughout, it was also attempted to correlate dye decolorization and incubation time. First it has been determined that whole culture of *C.versicolor* was able to decolorize Orange II and then decolorization ability of CCF of *C. versicolor* was investigated. CCF of *C. versicolor* was able to decolorize Orange II dye during trophophase parallel to biomass production and continued to decolorize in the later phases of development. An increase in the decolorization activity was determined in the secondary phase. The decolorization and degradation of various dyes by crude extracellular filtrate or directly and partly purified enzymes were demonstrated (1, 2, 5, 11, 12, 13). Greene and Gould (13) reported the decolorization activity of the filtered crude culture filtrate. In their study it was reported that decolorization activity of this filtrate was associated with a particulate body. Kim et al. (20) did not observed decolorization of Reactive blue 5 dye when the dye was mixed with crude enzyme solution of yeast like-fungus without H_2O_2. It was reported that biodegradation of dyes with the culture filtrate of ligninolytic fungi requires H_2O_2 or a H_2O_2 generating system (4, 13, 14). It has been shown here that CCF was able to decolorize various dyes without adding H_2O_2 and decolorization occured in nonligninolytic crude culture filtrates of the fungi. Chivukula and Renganathan (21) reported that laccase enzyme from *P. oryzae* was capable of oxidizing phenolic azo dyes. The fungi except *P. chrysosporium* produce laccase enzyme. Laccase and peroxidase appear to utilize similar mechanism for dye oxidation (21). It has been found recently that crystal violet can be degraded by commercial horseradish peroxidase (22).

This study showed that decolorization can be obtained by using directly crude culture filtrate of white rot fungi in the absence of exogeneously added H_2O_2 and higher rates of decolorization can be obtained by carefully selecting the metabolic phase of the fungi used. It has been also found that white rot fungi have different specificities towards dyes. Moreover, the crude culture filtrate of the white rot fungi may be employed in an efficient manner to decolorize the dyes.

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