Effect of Culture Parameters on the Decolorization of Remazol Brilliant Blue R by *Pleurotus ostreatus*

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The influences of culture parameters on the decolorization of anthron-type dye, Remazol brilliant blue R(RBBR) by *Pleurotus ostreatus* were studied in defined media. In the decolorization, 1~10 mM nutrient nitrogen and 40 mM glucose were effective whereas agitation and Tween 80 were not suitable. The decolorization occurred and the activity of extracellular peroxidase was detected during the stationary phase.

Key words: RBBR, decolorization, *Pleurotus ostreatus*

White-rot fungus, *Pleurotus ostreatus* secrets extracellular peroxidase and D-glucose oxidase which might play an important role in lignin degradation (8, 14). However, little has been known about the nutritional, physiological, and environmental factors that influence the biodegradation of lignin by this fungus. The major reason is that no unequivocal and sensitive quantitative assay for lignin degradation had been developed. Several studies have demonstrated that the efficiency of decolorization of certain dyes was correlated with the ability to degrade lignin compounds. Decolorization of a blue poly(vinylamine) sulfonate dye, Remazol brilliant blue R(RBBR), has been utilized by several groups of researches in identifying lignocellulose degradation abilities in fungi (16) and bacteria (12). Glenn and Gold (6) reported that three polymeric dyes, Poly B-411, Poly R-481, and Poly Y-606, served as alternatives to the radiolabeled lignin used as a substrate in lignin degradation assays. But, Ball *et al.* (1) reported a poor correlation between decolorization of the dye Poly R and ligninolytic activities of 20 actinomycetes. More correlation was found between lignocellulose weight losses and the ability to decolorize RBBR(11).

In this study, we examined the effects of several culture parameters on dye decolorization activity in *P. ostreatus* using RBBR.

Materials and Methods

Culture conditions

P. ostreatus was obtained from Korean Forest Research Laboratories, Korea. The fungus was inoculated in 500 ml Erlenmeyer flasks containing 100 ml of medium and cultured for 3 days as described previously (8). All flask contents were combined, aseptically separated from mycelial pellet by filtration through a Watman No. 1 filter paper, and washed 3 times on the filter with sterilized distilled water. For dye decolorizing experiments, the washed mycelia (2 g wet weight) were transferred to the minimal medium proposed by Commanday and Macy (3). Cultures were incubated for 10 days without agitation at 30℃. Agitated cultures were shaken at 150 rpm in a rotary shaker and Tween 80 was added at 0.05% (w/v) final concentration to the medium.

Decolorization assay

RBBR was added to the liquid medium as an aqueous solution to a final concentration of 0.02% with gentle swirling. Directly after its addition and at the indicated intervals, the culture medium was removed and diluted 5-fold with water. Decolorization was measured as the rate of decrease in the A_{592}/A_{500} ratio according to the method of Pasti and Crawford (11). UV-visible absorption spectra of dye were determined on a Shimadzu UV-3100 spectrophotometer (Japan).

Enzyme assay

An aliquot of extracellular culture medium was incubated in 3 ml of 20 mM sodium acetate (pH 4.0) containing 0.5 mM o-dianisidine and 0.1 mM H_2O_2. Its activity was determined at 35℃ by monitoring the absorbance

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Table 1. Effects of nitrogen sources on the decolorization of RBBR by *P. ostreatus*.

Nitrogen source	Concentration (mM)	A_{592}/A_{500}	Relative decolorization (%)
Ammonium chloride	1	0.38± 0.05	97.8
	10	0.36± 0.08	98.4
	100	1.02± 0.03	77.7
L-Glutamic acid	1	0.47± 0.11	95.0
	10	1.11± 0.36	75.0
	100	1.18± 0.08	72.8
Peptone	1	0.31± 0.04	100
	10	0.68± 0.34	88.4
	100	1.98± 0.18	47.8
Yeast extract	1	0.41± 0.02	96.8
	10	0.80± 0.29	84.6
	100	1.83± 0.07	52.5
Control		0.59± 0.14	91.2

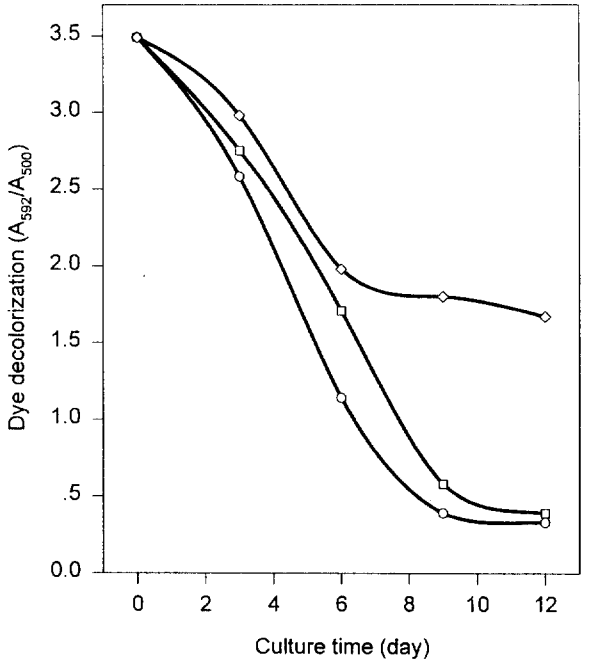

Fig. 1. Effect of nutrient nitrogen on RBBR decolorization. The dye was added to triplicate in the presence of 1 mM (○), 10 mM (□), and 100 mM (◇) NH_4Cl. As indicated, samples were removed and diluted, and the absorbance ratio were measured.

change at 430 nm. One unit(U) of enzyme was defined as the amount of enzyme required for an increase in absorbance of 1.0/min. Total protein was assayed by the Coomassie blue-dye binding method (2) with bovine serum albumin as the standard.

Chemicals

RBBR was purchased from Aldrich Chemical Co. (Milwakee, USA). Peptone (9.5% total nitrogen), yeast extract (11.0% total nitrogen), and malt extract were obtained from Difco Laboratories (Detroit, USA). All other chemicals were commercially available and were used without any further purification.

Table 2. Effects of carbon sources on the decolorization of RBBR by *P. ostreatus*.

Carbon source*	A_{592}/A_{500}	Relative decolorization (%)
Cellulose (CMC)	1.50± 0.09	69.2
Xylan	1.17± 0.09	82.3
Glucose	0.73± 0.10	100
Xylose	1.21± 0.09	80.7
Glycerol	1.48± 0.04	69.8
Control	1.54± 0.02	67.6

* The concentration of each nutrient carbon was 0.3%.

Results and Discussion

Effect of nutrient nitrogen

The effects of various nitrogen nutrients on the decolorization of RBBR after 10 days are shown in Table 1. The source of nutrient nitrogen did not significantly influence RBBR decolorization. Glutamate, which was known to be the most effective nitrogenous compound in suppressing ligninolytic activity (4, 5), did not have a significant suppressive effect on *P. ostreatus*. Peptone acted as the best nitrogen source. Although the source of nitrogen was not critical, the concentration of nitrogen greatly influenced the rate and extent of decolorization. For example, decolorization (A_{592}/A_{500}) after 9 days at 100 mM, 10 mM, and 1 mM NH_4Cl was 1.8, 0.6, and 0.4 respectively. 100 mM NH_4Cl suppressed dye decolorization at the level of 30~40% compared to 1 and 10 mM nitrogen (Fig. 1). Kirk *et al.* (9) reported that degradation of lignin by white-rot fungus *Phanerochaete chrysosporium* was suppressed by high concentration (24 mM) of nitrogen. Tonon *et al.* (15) postulated the existence of a regulatory gene mediating nitrogen catabolite repression similar to the *Aspergillus nidulans areA* gene and that nitrogen metabolism in *P. chrysosporium* was similar to that in *A. nidulans* in its regulation. Another white-rot fungus *Bjerkandera* sp. was distinct from *P. chrysosporium* and produced lignin peroxidase in nitrogen- sufficient glucose-peptone medium (7). Nitrogen suppression in *Pleurotus* was not so well-established. *P. ostreatus* produced an extracellular peroxidase that oxidized various phenolic lignin model compounds in nitrogen-sufficient medium (8). Commanday and Macy (3) mentioned ^{14}C-CO_2 production from ^{14}C-cornstover lignin in growth-limiting (1 mM) nitrogen medium was 3 times higher than in nitrogen rich medium. However, Leatham and Kirk (10) found ligninolytic activity in *P. ostreatus* was

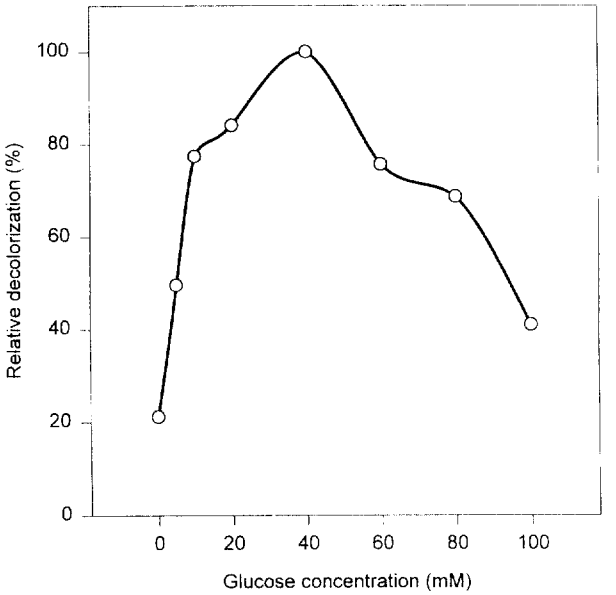

Fig. 2. Effect of glucose concentration on RBBR decolorization. Each culture contained indicated concentration of glucose and 0.02% dye. After 10 days cultivation, samples were removed, diluted, and the extent of decolorization were measured.

Table 3. Effect of culture condition and tween 80 on the decolorization of RBBR by *P. ostreatus*.

	Without shaking		With shaking	
	A_{592}/A_{500}	Relative decolorization (%)	A_{592}/A_{500}	Relative decolorization (%)
Tween 80	0.64± 0.09	99.3	3.09± 1.20	14.2
Controls	0.62± 0.14	100	2.36± 0.76	39.6

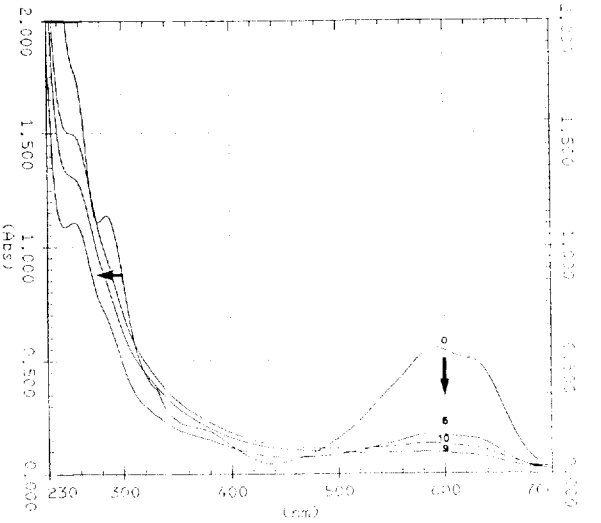

Fig. 3. UV-Visible spectra of RBBR according to culture age. Samples were removed as indicated day and diluted. The spectra were taken with a Shimadzu UV-3100 spectrophotometer.

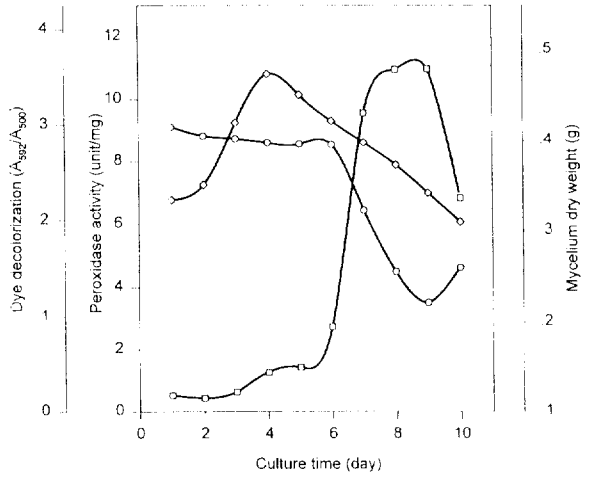

Fig. 4. Dependence of dye decolorization, growth rate, and peroxidase activity on culture age. Cultures were collected and the decolorization of RBBR (○), mycelium dry weight (◇), and extracellular peroxidase activity (□) were determined as described in the text.

not affected by nitrogen supplementation. It was possible to conclude that the nature of the native substrate used by ligninolytic fungi may influence whether the organism is susceptible to nitrogen suppression.

Effect of nutrient carbon

When several carbohydrates (0.3 % each) was added to the medium, glucose could serve as a carbon and energy source that could support dye decolorization (Table 2). Glycerol (69.8%) and cellulose (69.2%) did not support effective dye decolorization. The effect of concentration of glucose on dye decolorization was investigated. Increasing glucose concentration up to 40 mM resulted in an increase in the extent of dye decolorization. Decolorization rate and extent of RBBR was decreased above 40 mM of glucose (Fig. 3). Short-term depression of lignin degradation by high concentrations of glucose was found in *P. chrysosporium* (9), but stimulatory effect of excess carbohydrates had also been found in *P. chrysosporium* and *P. ostreatus* (3). Maybe excess carbohydrates promoted long-term ligninolytic activity, and high substrate C : N ratios yielded optimal activity.

Effect of agitation and detergent

Agitation had been widely reported to suppress both ligninase production and lignin degradation. Shimada *et al.* (13) had postulated that pellet formation due to agitation might have serious physiological effects in *P. chrysosporium* and could affect secondary metabolism. In contrast, Venkatadri and Irvine (17) had reported that agitation seemed to affect the catalytic activity of ligninase and had no apparent effect on either the rate of ligninase

production or the physiology of the fungus. Futhermore, the detergent, Tween 80 was able to protect both purified ligninase and extant ligninase in culture fluids against mechanical inactivation due to agitation. Our results showed that agitation seemed to suppress activity to about 60% that of static culture. Tween 80 had no apparent protective effect on dye decolorization in shaking culture (Table 3). It was possible that extracellular enzymes such as peroxidase of *P. ostreatus* were inactivated in agitated cultures and that detergent did not act as a mechanical protector in this fungus.

UV-visible spectra of culture fluid

Fig. 4. shows the change of absorption spectra of RBBR in culture filtrates of *P. ostreatus* with culture age. The absorbance decrease caused in major visible peak of the substrate around at 590 nm and hypsochromic shift was observed at UV region. When commercial horseradish peroxidase and H_2O_2 were incubated with RBBR in 0.2 M Tris buffer (pH 7.6), the spectrum of RBBR was changed similar to our results (11). Similar results have been obtained using an H_2O_2 generating system instead of H_2O_2. These results suggest that peroxidase enzymes could be involved in the decolorization process.

Dye decolorization and extracellular peroxidase activity

The time course of the decolorization of RBBR and extracellular peroxidase activity is shown in Fig. 5. Decolorization started after stationary phase, at approximately 6 days, and continued through the 9-day course of the experiment. Organism growth was essentially complete after 3 days, so it was concluded that RBBR decolorization, like ligninolytic activity, appeared to be a secondary metabolic process. Maximum extracellular peroxidase activity was detected at day 9. We reported that *P. ostreatus* produced one type of extracellular peroxidase in non-ligninolytic rich medium and did not decolorize RBBR in vitro (8). From these results, this fungus may produce another type of peroxidase. Further research will be necessary to purify and characterize this peroxidase responsible for dye degradation.

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