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Development of bioreactor systems for decolorization of Reactive Green 19 using white rot fungus

Ajeng Arum Sari[a,*], Sanro Tachibana[b], Muryanto[a], Tony Hadibarata[c]

[a]*Research Center for Chemistry, Indonesian Institute of Sciences, Kawasan Puspiptek Serpong, Tangerang Selatan, Banten 15314, Indonesia, Tel. +62 21 756 0929; emails: ajeng_as@yahoo.co.id (A.A. Sari), muryanto@lipi.go.id (Muryanto)*
[b]*Faculty of Agriculture, Department of Applied Bioscience, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan, Tel. +81 89 946984; email: tatibana@agr.ehime-u.ac.jp*
[c]*Institute of Environmental and Water Resource Management, Universiti Teknologi Malaysia, Skudai, Johor Bahru, 81310 Johor, Malaysia, Tel. +60 75531760; email: hadibarata@utm.my*

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ABSTRACT

The ability of *Trametes versicolor* U97 to decolorize Reactive Green 19 in bioreactor was investigated to determine whether the immobilized enzyme would be suitable for decolorization of Reactive Green 19 under agitation and nonagitation condition. Free cells of *T. versicolor* U97 showed an ability to decolorize Reactive Green 19 by approximately 44% in 72 h. Mediator mixture containing Tween 80, $MnSO_4$–H_2O_2, and hydroxybenzotriazole was added to the immobilized fungi to improve the decolorization process. Reactive Green 19 was decolorized by the immobilized fungi by approximately 80%, which is a twofold improvement over decolorization without mediators. In bioreactor system, decolorization of Reactive Green 19 was increased to 82% after 72 h. We evaluated the efficiency of the decolorization model for use in a small industry. Our results indicated that the wastewater discharge of 10 m^3 d^{-1} requires a reactor volume of 24 m^3 to obtain 80% decolorization with a retention time of 1.75 d. This study identifed that bioreactor of *T. versicolor* U97 immobilization is the most promising method for use in decolorize Reactive Green 19 and may be suitable for the treatment of wastewater in small industries.

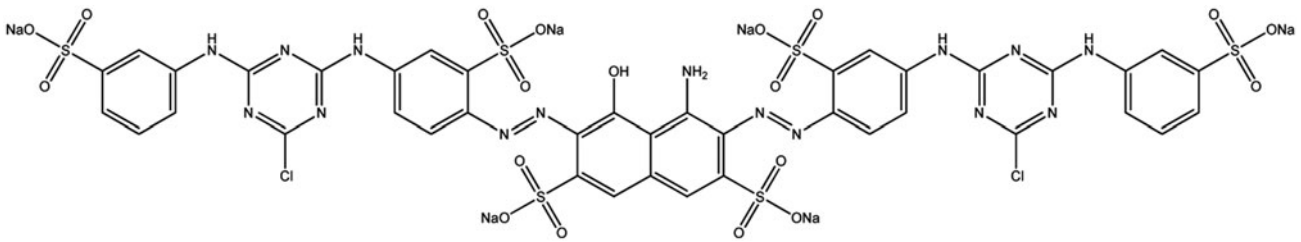
Keywords: Reactive Green 19; Decolorization; *Trametes versicolor*; Immobilized fungi; Immobilized enzymes; Bioreactor; Design large scale

1. Introduction

Synthetic dyes are used extensively in textile dyeing, paper printing, leather dyeing, and other applications. More than 2,000 different azo dyes are currently used to dye various materials and contaminate the environment through wastewater [1]. Azo dyes are a reactive chromogenic group of dyes that characterized by one or more azo groups (–N=N–). It exhibits a wide variety of colors and structures. These dyes are highly stable and resists microbial degradation [1,2]. Reactive Green 19, a sulfonated diazo reactive dye, has a complex chemical structure and a high molecular weight with two chromophoric azo groups and two reactive chlorotriazine groups (Table 1) [3]. This compound is commonly used in paint and garment industries in India [2]. The presence of azo dyes pose

*Corresponding author.

Table 1
Properties of Reactive Green 19

Parameter	Value
Chemical index name	Reactive Green 19
Functional group	Azo
CAS number	61931-49-5
Mol. mass (g mol^{-1})	1418.94
Mol. formula	$C_{40}H_{23}Cl_2N_{15}Na_6O_{19}S_6$
Chemical formula	
Lambda max	630
Appearance	Blue green powder
Solubility	120 g L^{-1} in water at 20°C
Manufacturer	Sigma–Aldrich
pH of the stock solution	5.5

a serious concern in effluent treatment plants due to their color, recalcitrance, potential toxicity to pristine ecosystems, total organic carbon, and chemical oxygen demand. It causes severe environmental problems worldwide [4]. Removal of azo dyes is difficult to degrade using typical wastewater treatment processes due to their stability and resistance towards light or oxidizing agents. Saratale et al. [4] reported that azo dyes and their metabolites are toxic, carcinogenic, and mutagenic in nature. They lead to the formation of tumors, cancers, allergies, and cause growth inhibition of bacteria, protozoan, algae, plants, and different animals. Azo dyes can cause immediate-type allergic reactions: urticaria, asthma, and rhinitis, and also allergic contact dermatitis [5]. For human life, the clinical and immunological investigation showed that 15% of 400 workers handling reactive dyes experienced work-related got respiratory and nasal symptoms [6]. Considering it's potential adverse effects on aquatic organisms, it is essential that this dye should be treated biologically before releasing into the effluent. One promising way is using micro-organism that secrete oxidizing enzymes to reduce the toxicity (Table 2).

The fungi used in this study are belonging to the genus *Trametes*, which can secrete ligninolytic enzymes and possible to decolorize azo dyes. Free cells of *Trametes* have been evaluated for their potential to decolorize several dyes. The *Trametes versicolor* U97 strain was found to degrade 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) and decolorize Remazol Brilliant Blue R most efficiently [7–9]. However, decolorization of dye using free cells is not conducive to large-scale optimization. Recently, immobilized fungi and immobilized enzymes systems have been developed to decolorize dyes [10]. The use of them offers various benefits over the use of free fungal cells, including protection of microbial cells from possible toxic pollutants and environment conditions, easy packaging, short retention time, and easy cell recovery [11–13]. However, the enhanced enzymatic activity of the immobilized fungi does not last long and may decrease overtime. On the other hand, although the stability of the enzyme depends on a composition and the chemical structure of the dyes, immobilized enzymes are more stable than free enzymes [1,14]. Previous study showed that some dyes such as Lefavix Blue 16, Reactive Remazol Violet 9, and Reactive Remazol Navy 4 were decolorized by immobilized enzyme of *T. versicolor* U97 in the range of 46–84% at pH 4.5 for 24 h [15]. However, the size and physical properties of the foam matrix may affect the decolorization process [5]. Redox mediators enhance the enzymatic oxidation of dyes and, therefore, have promising industrial applications [16]. However, a

Table 2
Comparison degradation of Reactive Green 19 by using several methods

Method	Initial concentration (ppm)	Degradation	Time (h)	References
Degradation by fungi *Pestalotiopsis* sp. NG007	100	98% by free cell 94% by immobilized fungi in bioreactor	72 24	[15]
Degradation by immobilized laccase using poly(4-vinylpyridine) grafted and Cu(II)chelated magnetic beads	50	77% 37% (after repeated 5 times)	18 18	[35]
Adsorption by fungi and yeast	50	*A. japonica* 7% *A. niger* 61% *R. arrhizus* 52% *R. nigcans* 86% *S. cerevisiae* 83% IRA 68 86% Activated charcoal 75%	All 1	[36]
Degradation by immobilized laccase from *Micrococcus glutamicus*	50	100%	42	[2]
Decolorization and degradation by *Emericella nidulans* TSF-12	1,000	94.52% at shaking condition	72	[32]

systematic effort to evaluate the potential of a bioreactor system to mediate oxidative reactions catalyzed by enzymes with the aim of identifying an economical, efficient, and eco-friendly process for decolorization of dyes as an environmental application has not been undertaken.

In this study, we evaluated the decolorization of Reactive Green 19 by free cells, immobilized fungi, and immobilized enzymes of the *T. versicolor* U97. The effect of mediators (Tween 80, $MnSO_4$–H_2O_2, and hydroxybenzotriazole [HBT]) on the decolorization under conditions of agitation, nonagitation, and in a bioreactor was also investigated. We evaluated the performance of the immobilized *T. versicolor* U97 fungi in a 4 L bioreactor.

2. Materials and methods

2.1. Decolorization by free cells of T. versicolor U97

T. versicolor U97 was cultured on a malt extract agar medium (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, agar 20 g L^{-1}, and polypeptone 1 g L^{-1}) at 25°C for several days. This strain was stored at a low temperature of 4°C. For preliminary analyses, *T. versicolor* U97 was used to decolorize Reactive Green 19 in a liquid medium. Three agar plugs of the fungus were inoculated into 20 mL of malt extract liquid medium (malt extract 20 g L^{-1}, glucose 15 g L^{-1}, and polypeptone 1 g L^{-1}) in 100 mL Erlenmeyer flasks. The inoculated flasks were preincubated for 7 d under static conditions at 25°C.

2.2. Preparation of immobilized fungi

The culture was homogenized at 10,000 rpm for 10 min after the preincubation. The crude fungi was mixed with 1.5% sodium alginate for bead preparation. The alginate–fungal mixture was then dropped into 0.1 M $CaCl_2$ (diluted in water). Three types of immobilized fungi (types I, II, and III) were tested in this study. Type I (immobilized without treatment) was prepared by mixing 20 mL of crude extract with 1.5% sodium alginate. Type II (immobilized double layer treatment) was prepared by mixing 20 mL of crude extract with 1.5% sodium alginate as the first layer. The bead was then coated with sodium alginate mixed with a supplement containing 1% Tween 80, 1 mM Mn^{2+}, 1 mM H_2O_2, and 0.5 mM HBT as the second layer. Type III (immobilized one layer treatment) beads were prepared by covering 20 mL of the crude extract with the supplement and 1.5% sodium alginate in a single layer.

2.3. Preparation of immobilized enzymes

T. versicolor U97 was grown on wood meal medium with supplementation (glucose 1.5% and a shiitake's sugar 2%) for approximately 30 d to prepare the immobilized enzymes. The culture was then extracted with 50 mM malonate buffer (pH 4.5) using a homogenizer at 10,000 rpm for 10 min, filtered to remove solids, and centrifuged at 8,000 rpm for 20 min at 4°C. The precipitate was discarded, and ammonium sulfate was added to the clear supernatant; the mixture was centrifuged at 8,000 rpm for 20 min at 4°C. The precipitate was resuspended in 50 mM malonate buffer and freeze dried prior to use. The crude enzyme (1.5 g; equal to 0.4 U mL^{-1} manganese peroxidase (MnP)) was used to produce the immobilized enzymes. Three types of immobilized enzymes were prepared as described in Section 2.2. The immobilized enzymes were stored at 4°C.

2.4. Reactive Green 19 decolorization culture and assays

Each flask was inoculated with free cells supplemented with 100 ppm of Reactive Green 19 in distilled water after preincubation. Distilled water containing Reactive Green 19 was used as a control. Then, solution of immobilized fungi and immobilized enzymes were supplemented with 100 mL of Reactive Green 19 (final concentration 100 ppm). Each reaction mixture was divided into three flasks and incubated under three different conditions: static, shaking (60 and 120 rpm), and in a bioreactor.

For the small-scale experiments, a glass column with a dead volume 0.06 L (working volume of 0.045 L) was used as a bioreactor. The immobilized fungi (35 g) and enzymes (35 g) were loaded onto the column. The column was filled with 100 mL of dye solution and operated continuously at a flow rate of 1 mL min^{-1}.

A glass column with a dead volume of 4 L was used as a bioreactor for the large-scale experiments. Type I-immobilized fungi (400 g) were loaded onto the column filled with 1 L of dye solution. The column was operated continuously with a rotary pump at a flow rate of 40 mL min^{-1}. The absorbance of Reactive Green 19 was determined using a UV–Vis spectrophotometer at intervals of 2, 6, 24, 48, and 72 h. Distilled water containing Reactive Green 19 was used as a control. The percentage of decolorization was calculated as follows:

$$\text{Decolorization}\,(\%) = \left(1 - \frac{C}{C_0}\right) \times 100 \quad (1)$$

C_0 is the initial dye concentration (ppm) and C is the final dye concentration (ppm).

The metabolites formed during the decolorization of Reactive Green 19 by the crude enzymes were analyzed at 0 and 24 h using a Waters 600E HPLC system (Milford, MA, USA). It was fitted with a Zorbax Eclipse XDB-C18 column with a particle size of 5 μm, id of 4.6 and a length of 150 mm. Optimum separation of the metabolites was obtained with a flow rate of 0.5 mL min^{-1}. The metabolites were detected spectrophotometrically at a wavelength of 585 nm. The eluent solvent consisted of acetonitrile and 30 mM acetic acid buffer at pH 4.5. The elution was performed based on the method described by Osma et al. with slight modifications [17]. The sample injection volume was 5 μL.

2.5. Enzyme activity

After incubation for several time, inoculate was filtered to obtain a supernatant. The activities of three enzymes such as laccase, MnP, and lignin peroxidase (LiP), were determined by measuring the absorbance of the supernatant with a UV–Vis spectrophotometer after incubation with the corresponding substrates at 20℃ for 1 min [8]. All values were expressed as U L^{-1}, defined as the amount of enzyme required to oxidize 1 μmol of substrate in 1 min.

2.6. Kinetic model for Reactive Green 19 decolorization

The reaction kinetics model of immobilized fungi and immobilized enzymes was used to determine the optimum system for the treatment of Reactive Green 19. Based on the results of the decolorization, a mathematical model was derived to simulate the reaction kinetics as shown in Eq. (2) where C is the concentration of Reactive Green 19 at time t (min), C_0 is the initial concentration of Reactive Green 19 at time $t = 0$, and b and m are two characteristic constants related to the reaction kinetics [18].

$$\frac{C}{C_0} = 1 - \frac{t}{m + bt} \tag{2}$$

To solve for the constants, Eq. (2) can be linearized to give:

$$\frac{t}{1 - C/C_0} = m + bt \tag{3}$$

The constants m and b were determined by plotting $t/(1 - C/C_0)$ vs. t, where straight line with an intercept of m and slope of b was obtained. The corresponding physical meaning of m and b can be determined by deriving an Eq. (2) to give:

$$\frac{C/C_0}{\mathrm{d}t} = \frac{t}{(m + bt)^2} \tag{4}$$

When t approaches zero, the slope of the original can be resolved as:

$$\frac{C/C_0}{\mathrm{d}t} = -\frac{1}{m} \tag{5}$$

The physical meaning corresponds to the initial rate of Reactive Green 19 in the process. Therefore, higher the $1/m$ value, the faster is the initial decolorization rate of Reactive Green 19. When t is long and approaching infinity, the reciprocal of the constant b is the theoretical peak of Reactive Green 19 decolorization, which can be derived from Eq. (6):

$$\frac{C_{t\to\text{infiniti}}}{\mathrm{d}t} = -\frac{1}{m} \tag{6}$$

Equilibrium absorption based on the weight of the fungal biomass can be calculated from the following equation, where q_{eq} is the equilibrium absorption by the fungal biomass, q_{t0} is the concentration of Reactive Green 19, q_t is the concentration of Reactive Green 19 at equilibrium, and X is the weight of the fungal biomass.

$$q_{eq} = \frac{q_{t0} - q_t}{X} \tag{7}$$

3. Results and discussion

3.1. Decolorization by T. versicolor U97

Reactive Green 19 has a maximum absorbance at 630 nm and characterized by a long conjugated π-system that links its two azo groups [19]. Free cells of *T. versicolor* U97 has shown 44% decolorization of Reactive Green 19 within 72 h. A significant increase in the activities of laccase (168 U L^{-1}) and MnP (59 U L^{-1}) in cells obtained after decolorization, indicated the involvement of these enzymes in decolorization process (Fig. 1). With increasing incubation time and the pH still in the range 4.0–4.5, the result was in line with results of Yanto et al.'s [15] that pH 4.0–5.0 was

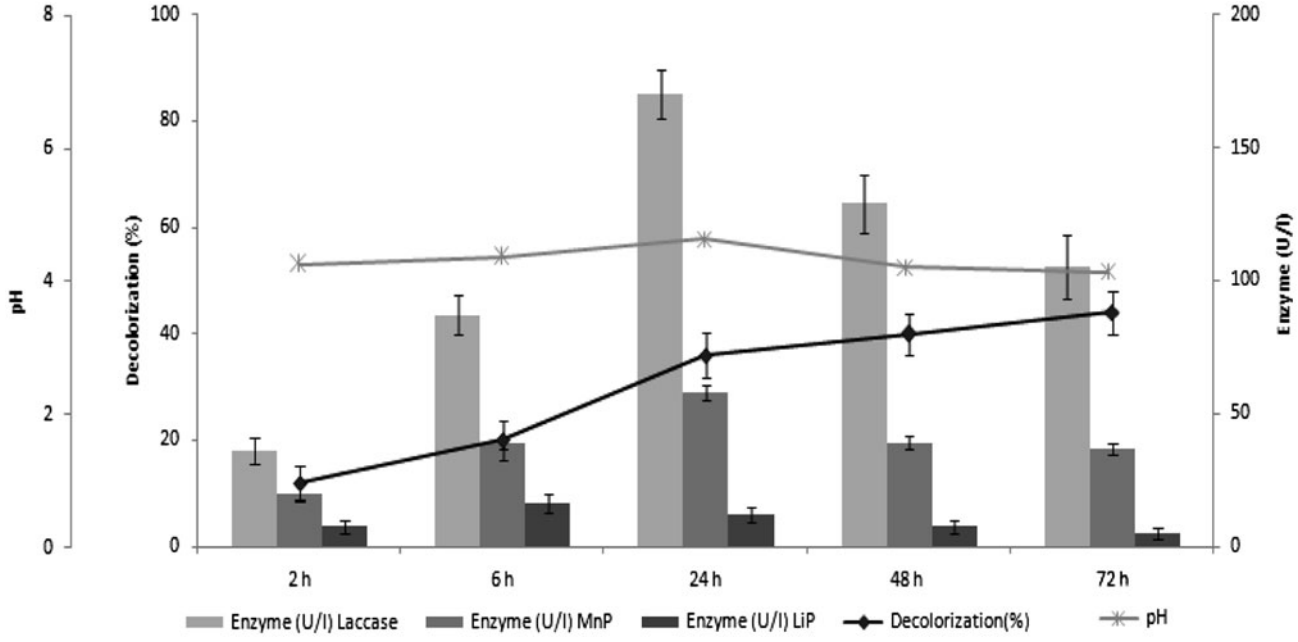


Fig. 1. Decolorization of Reactive Green 19 by free cells of *T. versicolor* U97 at 25˚C.

optimum pH for *T. versicolor* U97 to decolorize several dyes. The pH of the liquid culture can alter the fungal biomass surface charge, thus influenced the adsorption of the dye molecule to it [20]. Saratale et al. [4] showed that laccase as oxidoreductive enzyme can play a role for degradation of azo dyes. This enzyme can cooperate with MnP in dye decolorization when laccase indirectly produced H_2O_2 by oxidizing Mn^{2-} [21]. When laccase plays the primary role in decolorization, O_2 is required for decolorization. Laccase can regenerate the Mn^{3-}–organic acid complex and oxidize the dye molecule. The complexity of the structure of the azo dye is one of the main reasons for the difficulty in decolorizing Reactive Green 19. Azo dye decolorization begins with the breaking of the azo bond, the ease of which depends on the number and position of the functional groups in the aromatic region. Subsequently, the aromatic ring is cleaved. The rate of this step depends on the identity of the ring substituents and the presence of phenolic, amino, acetamido, 2-methoxyphenol, or other easily biodegradable functional groups [22]. Degradation of an azo dye Reactive Black 5 was initiated with cleavage of the azo bond by laccase. This result was investigated by Adnan et al. [23] that the degradation of this dye produces metabolites of 8-amino-naphthalene-1,2-diol and 4-sulfooxyethylsulfonyl-1-phenol, whereas for aromatic molecule phenylamine became sec-butylamine was mediated by MnP.

The implementation of the free cell culture system for the decolorization of Reactive Green 19 on a large scale is difficult, owing to exacting requirements such as the long incubation period necessary for fungal growth, sterility of culture media, supplemental nutrients, and adequate aeration [24]. In earlier studies, these limitations were overcome using immobilized enzymes, which have longer lifetimes [1].

Therefore, the efficiency of immobilized *T. versicolor* U97 cells and enzymes were tested for decolorization of Reactive Green 19 on a large scale. The immobilized fungi showed 38% dye removal in 24 h and 42% in 72 h (Fig. 2). It means that decolorization of dye by immobilized cells was slightly lower than by free cell. Immobilized *Trametes pubescens* and *Pleurotus ostreatus* showed 61 and 49% R243 dye removal in 48 h in low producing enzyme activity, respectively. The rate of decolorization was affected by the presence of a high portion of mycelium-associated enzyme the and simultaneous presence of isoforms with different affinities for the dyes and the reaction substrates during the decolorization process [25]. Furthermore, Husain [1] reported that the high rate of decolorization obtained with immobilized enzymes was due to the enzyme activity and high adsorption. However, our investigations revealed that less than 10% adsorption occurred, suggesting that the presence of sodium alginate did not interfere with decolorization and mycelia growth, and had a fast recovery time after exposure to the dye (Fig. 4) [26,27]. Surprisingly, in the absence of mediators, immobilized *T. versicolor* U97 enzymes in a batch system showed 20% decolorization in 72 h. Therefore, it was assumed that the low

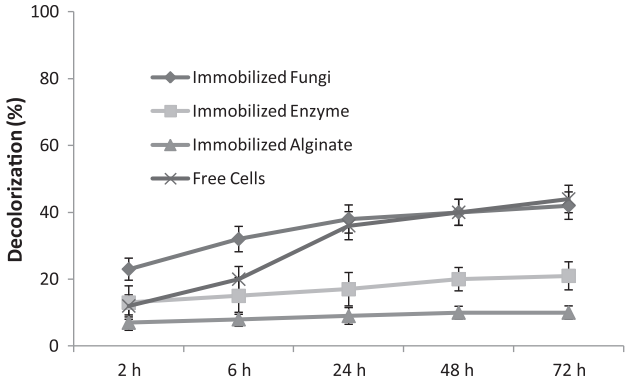


Fig. 2. Decolorization of 100 ppm Reactive Green 19 without mediators at 25°C.

rate of decolorization was due to the absence of other enzymes such as laccase and LiP, which may play important roles in the decolorization of Reactive Green 19. In addition, most immobilized fungi need mediators to enhance decolorization [1,28]. Laccase as the most dominating enzyme in this degradation is known to catalyze polymerization of azo dye degradation products over long periods of time where HBT can be used to favor polymerization these reactions and to elicit the destabilization of laccase [29]. The laccase-HBT system is more effective than laccase itself because redox potential of HBT is stronger than laccase. On the other hand, manganese ion is essential for a production of MnP in WRF [30]. Tween 80 was used to improve the bioavailability of pollutant compound. It provides an unsaturated fatty acid chain that may turn into a peroxyl radical for oxidation of dyes [29].

3.2. The effects of additional mixed mediators for decolorization

Immobilization technologies for redox mediators were conducted to overcome the limits of dissolved redox mediators which lost in a medium during dye transformation. In previous study, decolorization was optimized by the addition of several mediators to the immobilized fungi and enzymes. The addition of Tween 80, Mn(II), and veratryl alcohol increased the redox potential of MnP from *Phanerochaete chrysosporium* in a packed bed bioreactor and enhanced the decolorization of the azo dye, Astrazon Red FBL to 87% [30]. In our study, a mixed mediator consisting of, $MnSo_4$–H_2O_2, and HBT was added to the beads to enhance the decolorization process.

The mixed mediators in a double layer was added in the subsequent experiment. The results revealed that this design was not effective for interaction between the mediators and the enzymatic system of the fungi because the ability of the enzymes to decolorize the dye was inhibited by the sodium alginate in the second layer (data not shown). The decolorization of Reactive Green 19 by immobilized *T. versicolor* U97 with a single layer was improved by approximately twofold by agitating the reaction setup (Fig. 3(A)). A fourfold increase in decolorization was obtained when mediators was added to the solution to prevent the rapid inactivation of the enzymes during the degradation (Fig. 3(B)). Addition of Tween 80 in the presence of Mn^{2+} and H_2O_2 promoted the oxidation by MnP [15]. Tween 80 increase the ability of the dye and metabolites into mycelia or enzymes produced by the fungi.

Next, the effect of agitation on the decolorization of Reactive Green 19 was studied in greater detail. Immobilized *T. versicolor* U97 showed 85% decolorization of Reactive Green 19 at 60 rpm and 80% at 120 rpm, respectively (Fig. 3(A)). This decolorization level was 17% higher than that observed under nonagitation conditions. Agitation increased the interaction between Tween 80 and solution to enhance the mass transfer of oxygen, and provide adequate nutrients to

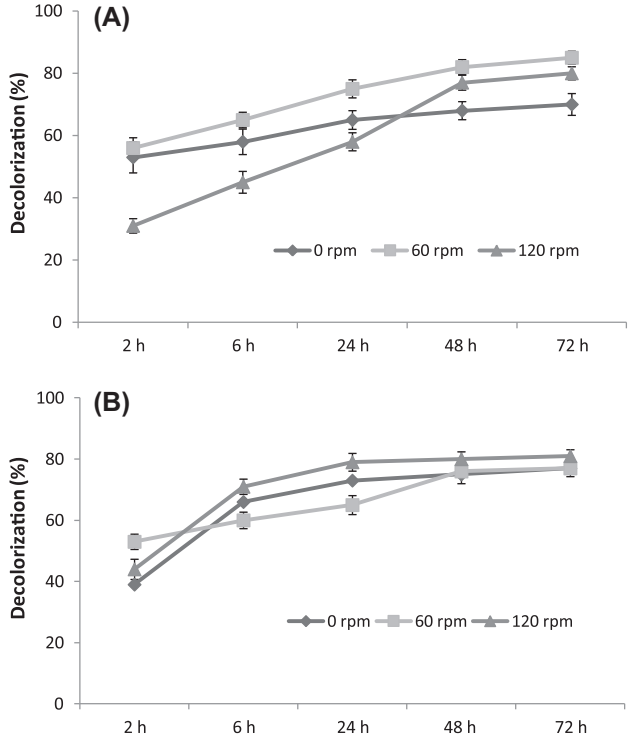


Fig. 3. Decolorization of Reactive Green 19 by adding mixed mediators: (A) immobilized fungi; (B) immobilized enzymes.

the micro-organisms [31]. Our results are consistent with those obtained by Shinde and Thorat who demonstrated that the level of decolorization at 150 rpm was slightly higher than that under nonagitation conditions [32]. However, high agitation conditions might lead to aerobic respiration by the microbes, which may dominate the utilization of NADH, and therefore, impede the electron transfer from NADH to the azo groups [2]. On the other hand, decolorization by the immobilized enzyme did not improve upon agitation at 60 and 120 rpm (Fig. 3(B)). Under these conditions, the oxygen transfer by immobilized enzyme may not be sufficient to increase decolorization.

Crude enzyme of *T. versicolor* U97 was used to detect the metabolite produced during decolorization of Reactive Green 19 using HPLC analysis. HPLC elution profile of the dye during the incubation period was changed, suggesting a change in the aromatic character of the initial dye [33]. In the HPLC analysis, a peak at a retention time of 8.7 min represented Reactive Green 19 solution at 0 h (Fig. 4(A)). After 24 h, several peaks corresponding to various metabolites

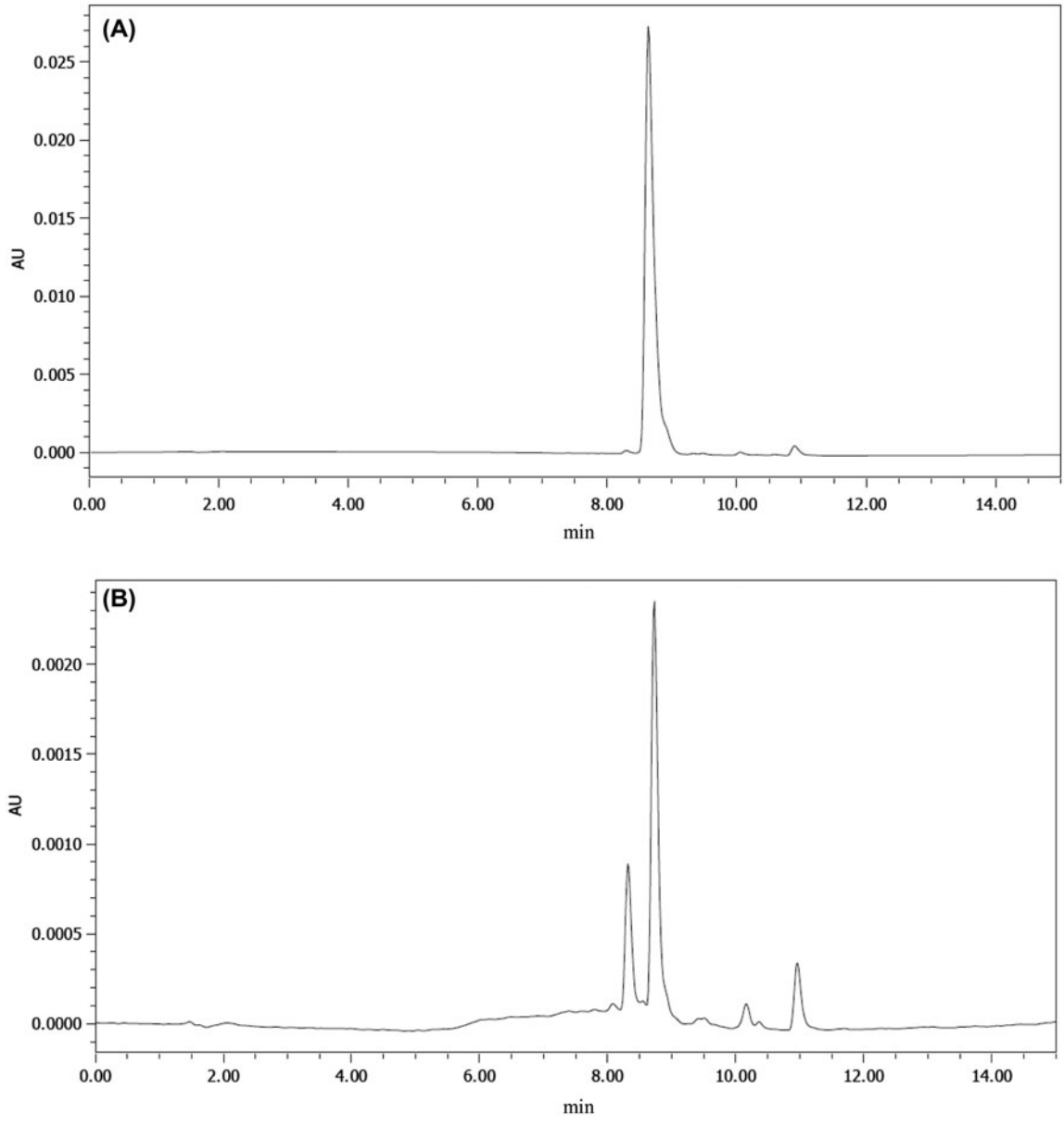


Fig. 4. HPLC profile during Reactive Green decolorization by crude enzyme of *T. versicolor* U97 (A) 0 h; (B) 24 h.

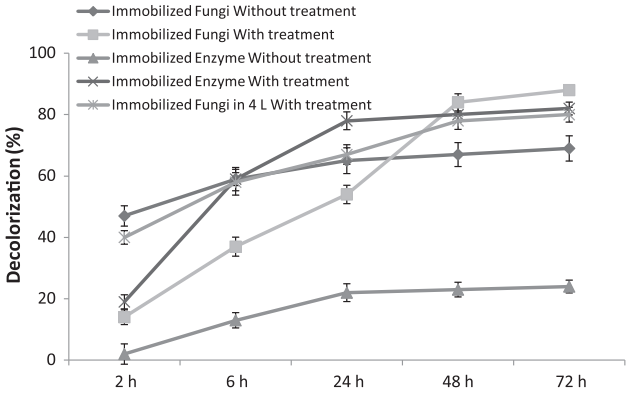


Fig. 5. Decolorization of Reactive Green 19 in a bioreactor by *T. versicolor* U97 at 25°C.

were observed at a lower retention time, indicating the formation of more polar oxidation products than the original dye (Fig. 4(B)) [33]. However, a detailed investigation to determine the identity of these metabolites of Reactive Green 19 decolorization is pending. Saratale et al. reported that the metabolite products identified during the decolorization of Reactive Green 19 were 2-aminobenzene sulfonic acid, 2-chloro-4,6-diamine-(1,3,5) triazine-3-amino benzene sulfonic acid; (3,4,6-triamino-5-hydroxy naphthalene-2,7-disulfonic acid); 2,4, diamino 6 hydroxy 1,3,5-triazine; benzene sulfonic acid; 8-amino naphthol; 1-naphthol; and naphthalene [2].

3.3. Application of decolorization using a bioreactor 0.045 L

In a bioreactor, immobilized fungi and immobilized enzymes showed 70 and 18% decolorization of Reactive Green 19 in the absence of any mediators in 72 h, respectively (Fig. 5). Decolorization of Reactive Green 19 was not improved by addition of the mixed mediator to the immobilized fungi solution. However, addition of the mixed mediator to the immobilized enzymes increased 72% decolorization. Enhanced decolorization in the bioreactor might be attributed to the higher rate of oxygen transfer and circulation, which resulted in a higher fungal growth rate and enzyme production. In the absence of mediators, the immobilized enzymes have no ability to decolorize Reactive Green 19 in a bioreactor as effectively as the immobilized fungi. Specific mediators related with producing MnP are needed to improve decolorization by the immobilized enzymes with a single layer to a level similar to that of decolorization by immobilized fungi.

3.4. A kinetic model and application of decolorization in bioreator 4 L

Table 3 shows the reaction kinetics of the decolorization of Reactive Green 19 by the immobilized fungi and enzymes. Maximum decolorization was obtained by treatment of immobilized fungi with a single layer. Immobilized fungi with a single layer was chosen as

Table 3
Reaction kinetics characteristic during decolorization of Reactive Green 19

Treatment			Maximum initial decay rate (mg g^{-1} min^{-1})	Predicted maximum decolorization (%)	Equilibrium absorption by fungal (mg g^{-1})
Immobilized fungi	Without treatment	Batch	−4.51	42.2	0.02
		Bioreactor	−13.13	69.2	0.04
	One-layer treatment	0 rpm	−12.78	70.2	0.04
		60 rpm	−9.76	85.5	0.05
		120 rpm	−3.39	82.6	0.05
		Bioreactor	−1.77	96.5	0.05
	Double-layer treatment	Batch	−3.62	38.5	0.02
		Bioreactor	−3.49	77.8	0.04
Immobilized enzyme	Without treatment	Batch	−1.66	21.1	0.01
		Bioreactor	−0.47	27.7	0.01
	One-layer treatment	0 rpm	−11.18	77.7	0.04
		60 rpm	−8.28	77.8	0.04
		120 rpm	−15.39	81.8	0.05
		Bioreactor	−4.65	85.5	0.05
	Double-layer treatment	Batch	−1.85	22.1	0.01
		Bioreactor	−0.42	48.0	0.02

Note: (−) means decolorization.

Table 4
Performance of decolorization with immobilized enzymes on a large scale

Characteristic	Amount	Unit
Input discharge	10	$m^3\ d^{-1}$
Pollutant concentration	100	ppm
Initial decay rate	(10.5)	mg g^{-1} min
m	95.24	–
Maximum decay rate	82.5	%
b	1.21	–
q_{eq}	0.02	mg g^{-1}
Assumed pollutant specific weight of dye	1,670	kg m^{-3}
Assumed biomass bulk density	777	kg m^{-13}
N = safety factor	1.2	–
Target degradation	80	%
Retention time	1.75	d
Maximum output discharge	10	$m^3\ d^{-1}$
Pollutant + water specific weight	1000.17	kg m^{-3}
Reactor volume	24	m^3
Minimum fungal biomass demand	100.02	kg m^{-3}

the best model for optimization in a large-scale setup. The maximum decolorization (82%) of Reactive Green 19 was reached after 72 h in a 4 L bioreactor (Fig. 5). It might be caused by small parts of immobilized in larger reactor having a contact with a dye and hence, a decreased rate of decolorization. However, the decolorization was stagnant after 48 h due to clogging of the bioreactor with mycelia, leading to a loss of aeration [34]. A simple design evaluation of decolorization performance with immobilized enzymes on a large scale was showed in Table 4. Our study suggested that a reactor volume of 24 m^3 is needed to obtain 80% decolorization of dye with a retention time of 1.75 d. Base on this study, an economic evaluation of the cost of decolorization should be performed to know the effectiveness of the process. However, the additional cost of the mediators must be considered during the evaluation. The economic feasibility of the process proposed in this report needs further investigation (e.g. disinfection and centrifugation).

4. Conclusion

Immobilized fungi and enzymes showed the ability to decolorize Reactive Green 19. Effective decolorization was obtained with the addition of a single layer a mixed mediator containing Tween 80, $MnSO_4$–H_2O_2 and HBT, under agitation. Immobilized *T. versicolor* U97 fungi decolorized the dye quickly and efficiently in a bioreactor. Bioreactor was an optimum method to enhance the decolorization of Reactive Green 19. A simple design evaluation revealed that a small industry with a wastewater discharge of 10 $m^3\ d^{-1}$ would require a reactor volume of 24 m^3 to obtain a decolorization of 80%. The kinetic model represented a practical importance in the use of *T. versicolor* U97 for decolorizing Reactive Green 19 on a commercial scale.

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