Decolorization of Direct Dyes Using Peroxidase from *Raphanus sativus* (F04 SL)

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(Received on 12th April 2011, accepted in revised form 1st November 2011)

Summary: An acidic peroxidase was isolated and partially purified from *Raphanus sativus*. The purified enzyme was characterized in terms of kinetics and thermodynamic aspects. Finally the enzyme was assessed to see its potential for decolorization of direct dyes. The specific activity of *Raphanus sativus* peroxidase increased from 44.77 to 65.20 U/mg of protein using 80 % ammonium sulphate precipitation. The optimum pH and temperature of the enzyme was 4 and 55 ºC respectively. The activation energy of *Raphanus sativus* peroxidase was 25.44 kJ/mol and average value of K_m was 0.25 mM. The activation energy of thermal denaturation of *Raphanus sativus* peroxidase was 17.79 kJ/mol. It was observed that with an increase in temperature, there was decrease in a half life and enthalpy, which showed that the enzyme was unstable at higher temperature. A maximum decolorization of 97 and 77 % was observed for Solar Blue A and Solar Flavine 5G at pH 4 and temperature 50 ºC respectively. It was observed that % decolorization of both the dyes increased with an increase in enzyme units and incubation time. H_2O_2 dose of 0.8 mM for Solar Blue A and 0.7 mM for Solar Flavine 5G was sufficient for the maximum dye degradation.

Keyword: Direct dyes; *Raphanus sativus*; Kenetics; Thermodynamics; Decolorization

Introduction

Peroxidase is an iron-porphyrin ring containing enzyme that belongs to class oxidoreductase. It generally catalyzes the redox reactions between H_2O_2 as an electron acceptor and different kinds of substrates by means of O_2 liberation [1]. Peroxidases are widely distributed in plants, microorganisms, and animals where these play important roles. However they are abundantly found in plants and have been involved in several physiological and biochemical processes such as participation in the lignification process [2], in the mechanism of defense in physically damaged or infected tissues and in the removal of toxic effects of hydrogen peroxide being produced during redox reaction [3]. Reduction of peroxides at the expense of electron donating substrates, make peroxidases useful in a number of biotechnological applications such as these may be successfully used for biopulping and biobleaching in the paper industry, and can produce oxidative breakdown of synthetic azo dyes [4]. Peroxidases have potential for soil detoxification. These enzymes have been applied for the bioremediation of wastewater and have also found use in biosensors and diagnostic kits. Enzyme linked immunosorbent assay (ELISA) tests on which peroxidase is probably the most common enzyme used for labeling an antibody, are a simple and reliable way of detecting toxins, pathogens, cancer risk in body and many other analytes [4]. However the novel applications of peroxidase include treatment of wastewater contaminated with dye because textile industry effluents are major source of water pollution due to stable nature of dyes towards light and temperature and have low biodegradability.

Dyes have a synthetic origin and complex aromatic molecular structure and are mutagenic and carcinogenic in nature. There are more than 10 000 commercially available dyes with annual production of over 7×10^5 tons [5]. However, about 10–15% of the synthetic dyes produced are discharged into industrial effluents [6] causing environmental problems. Conventional biological wastewater treatment systems, such as activated sludges, physicochemical treatments (flocculation, ozonation or membrane filtration) and microbial treatments are ineffective in dye removal because of high cost, low applicability and production of metabolites [7-11].

Recently, enzymatic approach has attracted much interest in the removal of dye from aqueous solutions [12-14] because enzymes can act on specific recalcitrant pollutants and the catalytic action of enzymes is extremely high as compared to chemical catalysts due to higher reaction rates and milder reaction conditions. They can catalyze reactions at relatively low temperature and in the entire aqueous phase pH range with low retention time [15, 16]. From a practical point of view the use of peroxidases in vitro for that purpose may represent a more feasible system, provided that a number of conditions are fulfilled: (a) a simple and applicable system; (b) minimal requirements of compounds; (c) low cost and stable enzyme; and (d) short treatment periods. This system could be implemented as a pre- or as a post-treatment for conditioning those concentrated fractions of effluents with strong color [8]. Keeping in view the significance of plant peroxidases the present study has been undertaken to

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investigate decolorization potential of the *Raphanus sativus* peroxidase (RSP) for direct dyes.

Results and discussion

Characterization of RSP

Plant leaves are rich source of peroxidase and several studies have demonstrated the ability of plant peroxidases to decolorize textile dyes. In the present investigation, peroxidase was isolated and purified from *Raphanus sativus* leaves. The partially purified enzyme was characterized in terms of kinetics and thermodynamic aspects. Finally the partially purified enzyme was used for the decolorization of direct dyes. In order to minimize the process cost simple ammonium sulphate precipitated proteins from RSP were used for degradation of dyes. Partially purified RSP was obtained by adding 80 % ammonium sulphate and exhibited a specific activity of 65.20 U/mg.

The results regarding the effect of pH on RSP activity are depicted in Fig. 1. The RSP was active within the pH range of 3-10. Maximum activity was observed at pH 4.0 which was optimum pH of peroxidase. This optimum pH showed that the peroxidase was acidic in nature. Our results are in accord with the earlier reported results in which the peroxidase isolated from hulls of glycine max var HH_2 exhibited maximum activity at pH 4.6 [17]. In order to check the effect of temperature, the enzyme was assayed at different temperatures (25 - 70 ºC) at optimum pH (4.0). The enzyme exhibited maximum activity at 55 ºC that was optimum temperature of the enzyme (Fig. 2). An optimum temperature of 55 °C has also been reported three turnip peroxidases [18]. The kinetic parameters i.e. Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of RSP were determined from Lineweaver-Burk plot of experimental data (Fig. 3). Calculated value for V_{max} was 90.90 U/min and average value of K_m was 0.25 mM. The K_m values of 0.70 and 0.42 mM have been reported for the two acidic fractions (C_2 and C_3) of turnip [18]. Our results indicated that RSP has better affinity for hydrogen peroxide as compared to turnip peroxidase. The activation energy of the partially purified RSP was 25.44 kJ/mol.

The RSP was subjected to various heat treatments (50-80˚C) at different time intervals and the denaturation constant s (K_d) and half life ($t_{1/2}$) of the enzyme was determined at each temperature (Fig. 4). The activation energy of thermal denaturation of RSP was 17.79 kJ/mol. From kinetic parameters, various thermodynamic parameters such as free energy (ΔG*) entropy (ΔS*) and enthalpy (ΔH*) changes for thermal denaturation were determined. The results are shown in the Table-1. It was observed that with an increase in temperature, there was decrease in a half life which showed that the enzyme was unstable at higher temperature. The decrease in enthalpy of thermal unfolding (ΔH*) with increase in the temperature also showed that the less energy is required for thermal unfolding at higher temperature. An increase in ΔG* values observed which indicated that RSP shows resistance against thermal unfolding/denaturation at higher temperature. A value of 101.4 and 103.4 kJ/mol has been reported for enthalpy (ΔH*) and free energy (ΔG*) of thermal denaturation of chick pea peroxidase at 65 °C respectively [19]. Our results clearly showed that RSP possessed high free energy (ΔG*) of thermal denaturation as compared to chick pea peroxidase which indicated that RSP showed more resistance towards unfolding at higher temperature. Thus, RSP could be used on industrial scale for dyes degradation and bioremediation purpopses.

Table-1: Kinetic and thermodynamic parameters for irreversible thermal denaturation of RSP

Temp. (K)	K_d (min^{-1})	$t_{1/2}$ (min)	ΔH° $kJmol^{-1}$	ΔG° $kJmol^{-1}$	ΔS° $Jmol^{-1}$ K^{-1}
328	0.0078	88.84	26.36	104.9	-239.7
333	0.0080	86.62	26.32	106.6	-241.7
338	0.0096	72.18	26.27	107.7	-240.9
343	0.010	69.3	26.23	107.6	-237.4
348	0.011	63	26.19	110.6	-242.5

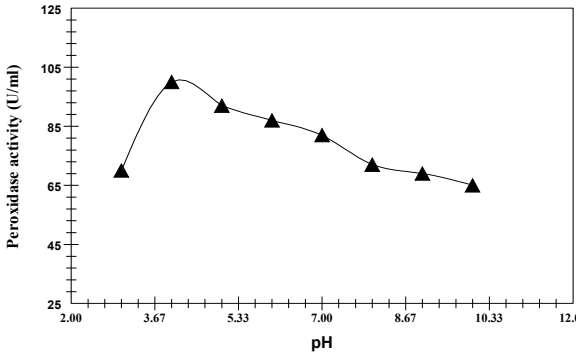

Fig. 1: Effect of pH on RSP activity.

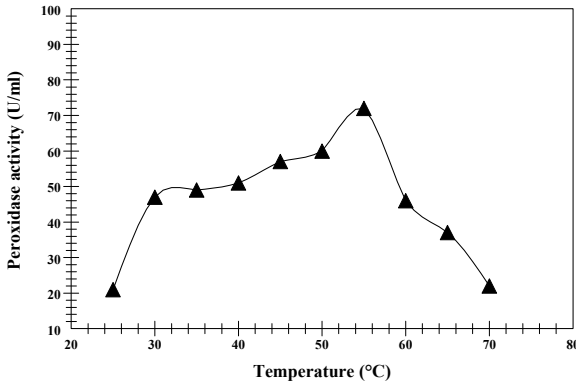

Fig. 2: Effect of temperature on RSP activity

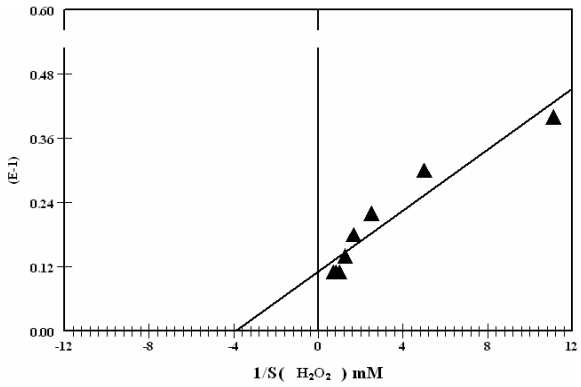

Fig. 3: Lineweaver- Burk plot for determination of V_{max} and K_m values of RSP

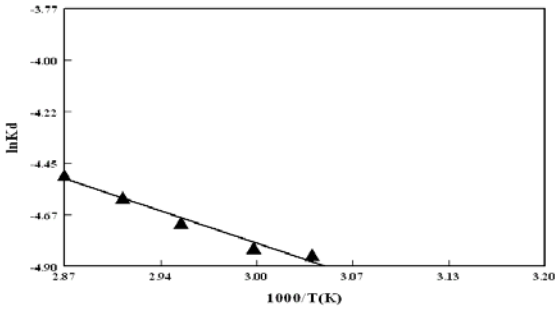

Fig. 4: Arrhenius plot for the determination of activation energy for thermal denaturation of RSP.

Decolorization of Direct Dyes

Optimization of various process parameters such as effect of peroxidase dose, pH of medium, temperature, concentration of hydrogen peroxide etc. was carried out using the classical approach of optimizing one parameter at a time. To study the optimum dose of RSP, experiments were carried out at various RSP doses ranging from 6-14 EU/mL at specified experimental conditions and the results are shown in Fig. 5. The RSP dose of 12 U/mL was found to be optimum dose for maximum dyes decolorization. The optimum enzyme dose of 2.205 EU/mL of horseradish peroxidase has been reported for maximum decolorization of acid azo (Acid Black 10 BX) at specified experimental conditions [20]. Satar and Husain [21] investigated the effect of white radish peroxidase (WRP) on the decolorization of reactive dyes. The results showed that decolorization of dyes was maximum in the presence of 0.4 EU/mL. The pH of medium is an important factor affecting dyes decolorization. The enzyme RSP showed a better performance (94 % and 77 % decolorization) at pH 4.0 for Solar Blue A and Solar Flavine 5G respectively (Fig. 6). With an increase in pH the decolorization efficiency was decreased indicating that RSP worked better at this pH. RR 120 and RB 171 were treated independently with soluble WRP and results revealed that optimal decolorization of both dyes occurred at pH 5.0 [21]. Similarly maximum decolorization of Remazol Turquoise Blue G and Lanaset Blue 2R by horseradish peroxidase has been reported at pH 4.0 [22].

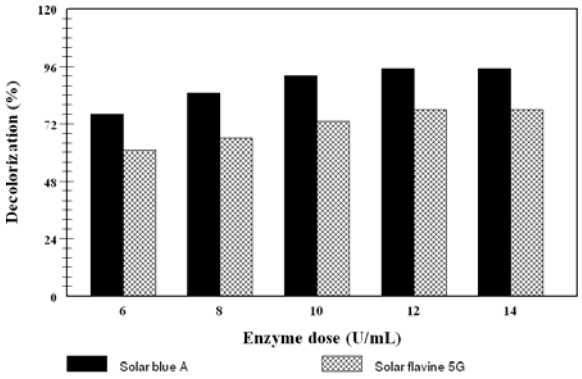

Fig. 5: Effect of RSP dose on the decolorization of direct dyes

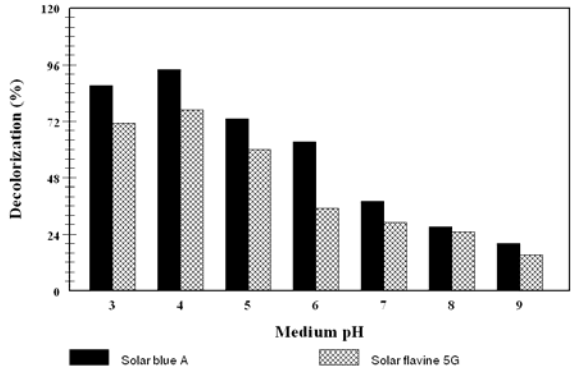

Fig. 6: Effect of medium pH on the decolorization of direct dyes by RSP.

Fig. 7 shows the effect of different temperatures on the decolorization of Solar Blue A and Solar Flavine 5G. The results showed that maximum decolorization of both dyes were recorded at 50 °C. Above and below the temperature the rate of decolorization was decreased. The decrease in dyes decolorization at higher temperature might be due to the denaturation of enzyme at higher temperature which resulted in low activity and hence low decolorization. Below the optimum temperature, the enzyme might not be able to achieve its energy of activation for reaction with the dyes, hence again low decolorization was observed. Maximum decolorization of acid dyes has been reported by salt fractionated turnip peroxidase at pH 5.0 and temperature 40 °C [23]. Similarly maximal decolorization of RR120 and RB 171 was observed at 40 °C [21]. The data regarding the effect of H_2O_2

concentration on the decolorization of direct dyes by RSP was shown in Fig. 8. From the data, it is evident that H_2O_2 doses of 0.8 mM and 0.7 mM were sufficient for enhanced decolorization of Solar Blue A for Solar Flavine 5G respectively. Maximum decolorization of Remazol Turquoise Blue G and Lanaset Blue 2R by horseradish peroxidase has been reported with H_2O_2 concentration of 2×10^{-3} mmol/L [23]. The rate of dye decolorization by peroxidases depends on the nature of dye and source of peroxidase. Similarly the dose of mediator also varies from dye to dye and source of peroxidase. Our results are contrary to the reported results in which low dose/concentration of H_2O_2 has been used for particular dyes.

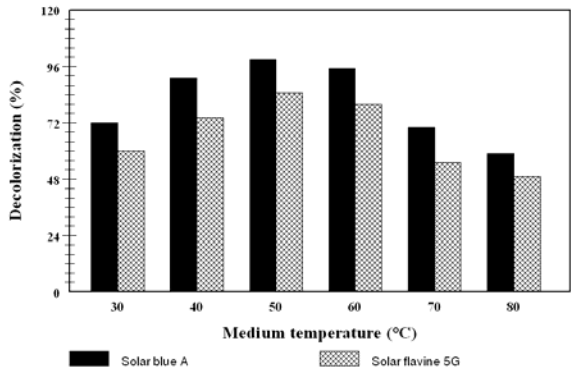

Fig. 7: Effect of temperature on the decolorization of direct dyes by RSP.

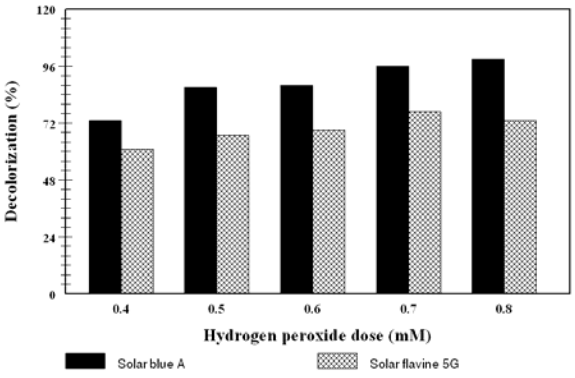

Fig. 8: Effect of H_2O_2 concentration on the decolorization of direct dyes by RSP.

Another important process parameter is the time of incubation. The efficiency of the decolorization of the direct dyes as a function of contact time with the enzyme is given in Fig. 9. It is evident from the figure that 40 minute of the reaction time is sufficient for the maximum dyes removal/decolorization of both the dyes. Optimum time of 45 minutes has been reported for maximum decolorization of the dye Acid Black 10BX. [20]. Similarly WRP has been reported to decolorize Reactive Red 120 (665 %) and Reactive Blue 171(65 %) after incubation for 1 h. This indicated that RSP required less time for decolorization of dyes. Finally the effect of dye concentration was optimized for both the dyes. Studies were carried out using different concentration of the dyes 20-100 ppm, keeping all the other parameters constant and the results are shown in Fig.10. The results indicated that maximum decolorization of both the dyes was observed with 20 ppm. Higher concentration of both the dyes resulted in decreased decolorization of the dyes.

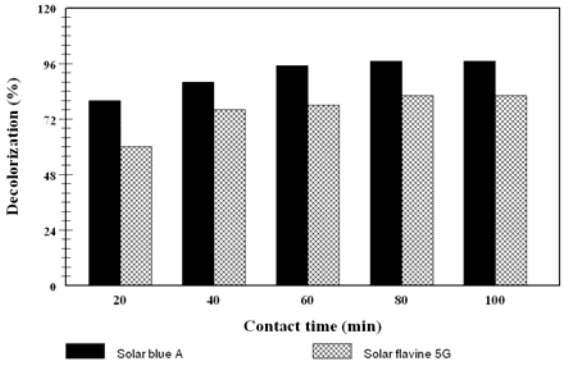

Fig. 9: Effect of contact optimum time on the decolorization of direct dyes by RSP.

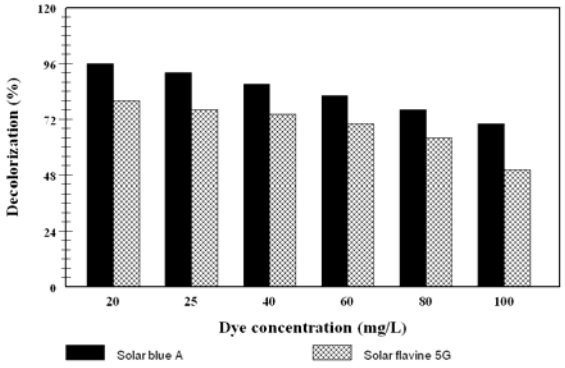

Fig. 10: Effect of dye concentration on the decolorization of direct dyes by RSP.

Experimental

All the chemicals and reagents used in the present study were of analytical grade and mainly purchased from Sigma, Chemical Company, USA, unless otherwise mentioned. Raddish (*Raphanus sativus*) leaves of a local cultivar, were collected from a local Vegetable Form, and stored at 4°C.

Peroxidase Extraction

Raddish leaves were collected from local vegetable market and washed thoroughly with distilled water to remove the surface dust. Then raddish leaves were homogenized with a blender in extraction buffer (20 mM sodium phosphate, pH 6.8) at a ratio of 20 mL extraction per gram of tissue. The homogenate was filtered through a filter paper [17].

Protein and Peroxidase Assay

Total proteins were estimated by following the Bradford method using bovine serum albumin (BSA) as the standard [24]. Peroxidase activity was determined with a spectrophotometer following the formation of tetraguaicol (A_{max} = 470, € = 26.6$mM^{-1}cm^{-1}$) in a 3ml reaction mixture containing 1mL buffer (0.1M acetate buffer pH 5), 1mL H_2O_2 (1.6mM), 1mL guaicol (15mM) and 50µL enzyme extract [17]. One unit of peroxidase activity (U) was defined the amount of enzyme catalyzing the oxidation of 1 µ mole of guaiacol in 1 min.

Partial Purification RSP

The crude extract was brought to 80 % saturation with solid ammonium sulphate by adding 520 g of ammonium sulphate in 1L of enzyme extract, stand overnight at 4 °C. Then centrifuged at 10000 rpm for 20 minutes [19]. The supernatant was separated carefully and precipitates were dialyzed extensively against four-five changes of distilled water to remove the salts. The eluates containing peroxidase activity were pooled and assessed for kinetic and thermal characterization.

Determination of Kinetic Constants

The effect of pH on RSP activity was determined by assaying the enzyme using buffers (acetate, phosphate, glycine/HCl and Tris/HCl) of different pHs ranging from 3 – 10 at 30°C. The optimum temperature of the enzyme was determined by incubating the enzyme in 0.1M acetate buffer (pH 5.0) at different temperatures ranging from 25 to 70°C [19]. The activation energy was determined by assaying peroxidase at various temperatures ranging from 25-70ºC and then by ploting data to Arrhenius plot. The Effect of substrate was determined by using the different concentration of H_2O_2 ranging from 0.09 to 1.4 mM keeping enzyme concentration constant. Line Weaver Burk plot was plotted between inverse of substrate concentration in mM and inverse of velocity, taking [1/s] along x-axis and [1/v] along y-axis. From the graph the values of V_{max} and k_m (Michealis-Menton constant) were calculated.

Kinetics and Thermodynamics of Thermal Inactivation

The RSP was subjected to various heat treatments (50-80˚C) at different time and the first-order rate constants for irreversible thermal denaturation (K_d) of peroxidase was determined and then Arrhenius plot was applied to determine the activation energy for denaturation (Ea). The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory [25].

$$k_d = (k_bT/h)\, e^{(-\Delta H^*/RT)} .e^{(\Delta S^*/R)} \quad (1)$$

where:

h = Planck's constant = 6.63 x 10^{-34} Js
k_b= Boltzman's constant (R/N) = 1.38 x 10^{-23} JK^{-1}
R= gas constant = 8.314 JK^{-1} mol^{-1}
N= Avogadro's No. = 6.02 x 10^{23} mol^{-1}
T= Absolute temperature
ΔH* (enthalpy of activation)

$$= Ea - RT \quad (2)$$

ΔG*(free energy of activation)

$$=- RT \ln (k_d\, h/k_b.T) \quad (3)$$

ΔS*(entropy of activation)

$$= (\Delta H^* - \Delta G^*)/T \quad (4)$$

Energy of activation (Ea) for thermal inactivation was determined from Arrhenius plot (Fig. 5).

Decolorization of Direct Dyes

The dyes (25 mg dye/l) solutions were prepared in 100 mM sodium acetate buffer, pH 4. Each dye was incubated with increasing concentration of RSP ''6–12 EU of RSP/ml of reaction volume'' in 100 mM sodium acetate buffer, pH 4 in the presence of 0.7 mM H_2O_2 for 40 min. at 40°C. After incubation, the treated dyes were centrifuged at 2000 rpm. Dye decolorization by RSP was monitored at each specific Wavelength. The percent decolorization was calculated by taking untreated dye solution as control (100 %). Experimental assays and controls were run in duplicate. Each dye was incubated with RSP (10 U mL^{-1}) in 100 mM sodium acetate buffer, pH 4 in the presence of 0.7 mM H_2O_2 for 40–100 min. at 40°C. After incubation, the treated dyes were centrifuged at 2000rpm and percent decolorization was calculated as mentioned before [20]. The effect of pH on dyes was investigated by incubated the dyes with RSP (10

U $mL^{-1)}$ in the buffers of various pH values ranging from 3.0 to 9.0 at specified experimental conditions and and percent decolorization was calculated [22].

In order to get optimum temperature for maximum decolorization, each dye was incubated with RSP (10 U mL^{-1}) in 100 mM sodium acetate buffer, at various temperatures 20–80 °C keeping all other parameters constant and percent decolorization was calculated. [23]. The effect of hydrogen peroxide concentration studied by incubating the dyes with RSP (10 U mL^{-1}) in 100 mM sodium acetate buffer (pH 4) with different concentrations of H_2O_2 ranging from 0.7 to 0.8 mM. at specified experimental conditions and percent decolorization was calculated[22]. Similarly the dyes concentration were optimized using preoptimized conditions [26-28].

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