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Decolourization of Drimarene Orange KGL by Orange (*Citrus reticulata*) Peroxidase

NOSHEEN HAFIZ[1], HAQ NAWAZ BHATTI[1,*], SHAZIA NOUREN[1] and MAJID MUNIR[2]

[1]Department of Chemistry, University of Agriculture, Faisalabad-38040, Pakistan
[2]Department of Chemistry, Government College University, Faisalabad, Pakistan

*Corresponding author: Fax: +92 41 9200764; Tel: +92 41 9200161-69/3319; E-mail: hnbhatti2005@yahoo.com; haq_nawaz@uaf.edu.pk

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Reactive dyes are widely employed in textile industries and their removal from wastewaters is a relevant environmental problem. In addition to chemical and physical methods, several bioremediation approaches, involving intact micro-organisms or isolated enzymes have been proposed to decolourize dye solutions. The aim of this study was to evaluate the enzymatic action of partially purified orange (*Citrus reticulata*) peroxidase for the degradation of industrially important dye Drimarene orange KGL. Studies were carried out to understand the process parameters such as aqueous phase pH, temperature, time, H_2O_2 dose, dye and enzyme concentrations during enzyme-mediated dye degradation process. Experimental data revealed that 81.36 % degradation of 60 ppm Drimarene orange KGL solution was obtained within 160 min in the presence of 33 U/mL of orange peroxidase, 2 mM H_2O_2 at pH 4 and 60 °C temperature.

Keywords: *Citrus reticulata*, Drimarene orange KGL, Specific activity, Ammonium sulphate, Degradation.

INTRODUCTION

Treatment of synthetic dyes in wastewater is a matter of great concern now-a-days. Several physicochemical methods have been used for the removal of dyes[1]. However, these procedures have not been widely employed due to their high cost, formation of toxic by products and intensive energy requirement[2]. Extensive research has been directed towards developing processes in which enzymes are being employed to remove dyes from polluted water[3-7]. The potential advantages of enzymatic treatment as compared to microbial include, shorter treatment period, operation of high and low concentrations of substrates, absence of delays associated with the lag phase of biomass, reduction in sludge volume and ease of controlling the process[6,8].

Peroxidases (E.C. 1.11.1.7), representing a huge family of heme containing enzymes are widely distributed in plants, microbes and animal tissues[9]. Plant peroxidases are found in tonoplast and plasmalemma, inner and outer side of the cellular wall both in soluble as well as ionically bound forms[10]. They oxidize several substrates in the presence of hydrogen peroxide and usually contain a protoporphyrin IX prosthetic group and have various physiological roles in plant cells. Peroxidases can act on specific recalcitrant pollutants by either precipitation or transformation into other products[4]. One of the commercial sources of plant peroxidase is horseradish peroxidase which has been utilized for its ability to decolourize lignin containing pulp and paper waste water as well as textile waste water[11]. The relation between the biodegradability and molecular structure of the dye is very important and it depends on the type, quantity and position of the substituted groups on the aromatic ring and the molecular weight of dyes[12].

Citrus represents one of the most important and widely grown crops in the world and Pakistan is one of the major citrus producing countries. As the focus of most of today's research is to explore economical sources so use of leaves of orange (*Citrus reticulata*) plant would be encouraged in this respect.

EXPERIMENTAL

Drimarene orange KGL was taken from a local textile industry while orange (*Citrus reticulata*) leaves of a local cultivar were collected from Citrus garden, University of Agriculture, Faisalabad. All the other chemicals and reagents used in the present study were of analytical grade and mainly purchased from Sigma, Chemical Company, USA, unless otherwise mentioned.

Extraction and partial purification of peroxidase: Fresh orange leaves (10 g) were thorougly washed with distilled water and then homogenized with 100 mL of pre-cooled 0.1 M phosphate buffer pH 7. The mixture was immediately filtered through the four layers of Muselin cloth. The filtrate was then subjected to 80 % ammonium sulphate fractionation by overnight continuous stirring in cold. The precipitated proteins were then collected by centrifugation at 10,000 rpm

for 15 min at 4 °C in a Semi Cooling Centrifuge C-24 and the pellets obtained were then redissolved in 25 mL of 0.1 M phosphate buffer (pH 7) and then subjected to dialysis against 0.025 M phosphate buffer (pH 7)[13,14].

Protein and peroxidase assay: Total proteins were estimated by Bradford micro assay using bovine serum albumin (BSA) as the standard[15]. Peroxidase activity was determined at 30 °C with a spectrophotometer (Hitachi model U-2001) following the formation of tetraguaiacol (A_{max} = 470 nm, ε = 26.6 mM^{-1} cm^{-1}) with slight modification[16]. The reaction mixture contained 1 mL of 0.1 M acetate buffer (pH 5), 1 mL of 15 mM guaiacol, 1 mL of 1.6 mM H_2O_2 and 50 µL of enzyme extract. One unit of peroxidase activity (U) was defined the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min.

Effect of enzyme dose for decolourization of drimarene orange KGL: To study the effect of enzyme dose on the decolourization process, 40 ppm dye solution prepared in 0.1 M buffer of pH 4 was incubated with increasing doses (3-39 U/mL) of enzyme in the presence of 1 mL of 1.25 mM H_2O_2 for 45 min at 35 °C. All the samples were centrifuged prior measuring the absorbance of drimarene orange KGL at the specific wavelength (λ_{max} 400 nm). The percent decolourization was calculated by using the untreated dye solution as a control (100 %).

Effect of pH for decolourization of drimarene orange KGL: The effect of pH on decolourization was investigated by incubating the dye solutions (40 ppm) prepared in different pH buffers ranging from pH 3 to 9 with 33 U/mL of enzyme and 1 mL of 1.25 mM H_2O_2 for 45 min at 35 °C. The treated samples were centrifuged before monitoring decrease in the absorbance at λ_{max} of drimarene orange KGL. The controls were also prepared in buffers and used for the calculations of per cent decolourization.

Effect of concentration of hydrogen peroxide for decolourization of drimarene orange KGL: The effect of hydrogen peroxide concentration was studied by incubating the drimarene orange KGL (40 ppm) with orange peroxidase (33 U/mL) in the presence of different concentrations of H_2O_2 ranging from 0.25-4.0 mM at 35 °C for 45 min. The absorbance of the dye solution at λ_{max} was recorded against untreated dye as control (100 %) and percent decolourization was calculated against untreated dye solution.

Effect of concentration of dye for decolourization of drimarene orange KGL: In order to investigate the effect of dye dose different concentrations of dyes were incubated with orange peroxidase (33 U/mL) in presence of 2 mM of H_2O_2 by keeping all the parameters as constant under the same experimental conditions. The absorbance untreated dye was taken as control (100 %).

Effect of temperature for decolourization of drimarene orange KGL: 60 ppm drimarene orange KGL in pH 4 buffer was incubated with orange peroxidase (33 U/mL) at different temperatures (35 to 70 °C). Other reaction conditions were common. The absorbance of the dye solution at λ_{max} was recorded against untreated dye as control (100 %) and percent decolourization was calculated against untreated dye solution.

Effect of time of incubation for decolourization of drimarene orange KGL: The effect of time of incubation was studied by incubating 60 ppm dye solution at different time intervals ranging from 15-200 min by keeping other parameters as constant under the same experimental conditions as mentioned earlier. The absorbance of untreated dye was taken as control (100 %).

Calculation of percent dye decolourization: The per cent dye decolourization was calculated for each dye. This parameter is defined as:

$$\% \text{ Decolourization} = A_u\text{-}A_t/A_u \times 100$$

where, A_u is absorbance of the untreated dye, A_t is absorbance after treatment.

Statistical analysis: The results were reported as mean ± standard deviation.

RESULTS AND DISCUSSION

To our best of knowledge, we have first reported the application of partially purified orange peroxidase extracted from leaves of the orange plant in the decolourization of an industrially important dye, drimarene orange KGL. In order to reduce the cost of the process, simple ammonium sulphate precipitated proteins from orange leaves were taken for the treatment of drimarene orange KGL. Partially purified preparation of orange peroxidase was obtained by adding 80 % ammonium sulphate and this preparation exhibited a specific activity of 70.45 U/mg of protein. Further experiments were designed to assess dye decolourization using the classical approach of optimizing one parameter at a time.

Optimum enzyme dose for decolourization of drimarene orange KGL: Normally removal of the aromatic compound is dependent on the amount of catalyst added since the catalyst has a finite lifetime and also the conversion is found to be dependent on the contact time. There is an optimum relationship between the concentration of enzyme and substrate for achieving maximum activity. So, experiments were conducted in which drimarene orange KGL was treated with increasing concentration of orange peroxidase and results are shown in Fig. 1. The percentage of dye decolourization was continuously enhanced with increasing the amount of enzyme. However, it was found that 33 U/mL of orange peroxidase was sufficient to decolourize 40 mg/L of drimarene orange KGL. After the optimum enzyme dose there was no increase or decrease in decolourization was recorded. Previously, similar trend was reported for decolourization of acid black 10 BX[17] and acid blue[21] both by horseradish peroxidase[18].

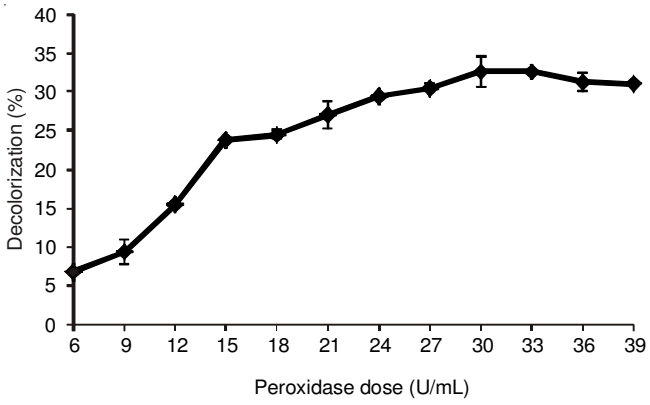

Fig. 1. Effect of the enzyme dose on the decolorization of the drimarene orange KGL

Optimum pH for decolourization of drimarene orange KGL: Enzymes have a characteristic pH at which they show maximum activity, the optimum pH depends on the nature of substrate being used[19-20]. Fig. 2 represents the effect of pH on the decolourization of drimarene orange KGL by orange peroxidase. The results reveal that the dye was maximally decolourized in acidic medium *i.e.*, pH 4. After this pH a continuous decrease in percent decolourization was recorded. This might be due to inactivation of the enzyme in basic medium. The same optimum was obtained in case of decolourization of Reactive Red 2, Disperse Black 9, Reactive Blue 4, Reactive Black 5 and Disperse Orange 25 with cauliflower bud peroxidase[21].

The result is also consistent with several earlier reports where acidic medium was considered best for the decolourization of dyes by various plant peroxidases. For example; horseradish peroxidase[3] and soybean peroxidase[22] showed maximum decolourization in acidic buffers, respectively for ramazol blue and remazol tuquise blue G 133.

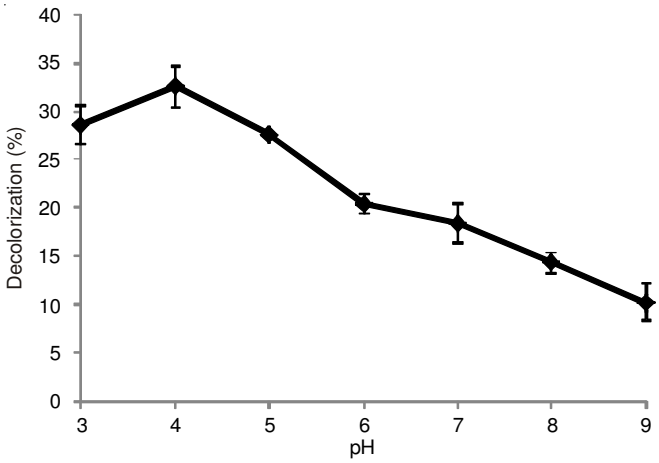

Fig. 2. Effect of the pH on the decolorization of the drimarene orange KGL

Optimum concentration of hydrogen peroxide for decolourization of drimarene orange KGL: Hydrogen peroxide acts as a co-substrate in order to activate the enzymatic action of peroxidase radical. It contributes in the catalytic cycle of peroxidase, to oxidize the native enzyme to form an enzymatic intermediate which accepts aromatic compound to carry out its oxidation to a free radical form. The effect of H_2O_2 on dye decolourization is represented in Fig. 3. It can be shown that 2 mM of H_2O_2 was sufficient for maximum decolourization of drimarene orange KGL, above this concentration a decrease in decolourization was reported. It means high concentration of hydrogen peroxidase showed an inhibitory effect. Comparing with previous reports less H_2O_2 was required for decolourizatioin purpose in most cases *e.g.* decolourization of direct dyes by Rapanus sativus peroxidase increased by increasing H_2O_2, although maximum decolourization was obtained at a concentration of 0.8 mM[23].

Optimum concentration of dye for decolourization of drimarene orange KGL: Concentration of the substrate has significant effect on any enzyme catalyzed reaction. Fig. 4 showed the effect of drimarene orange KGL concentration on % decolourization of dye. The dye decolourization was

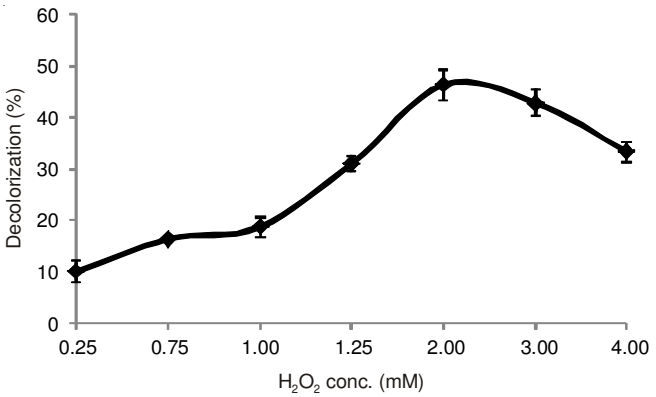

Fig. 3. Effect of the H_2O_2 concentration on the decolorization of the drimarene orange KGL

found to be effective up to 60 ppm of dye concentration. Further increase in dye concentration resulted in low dye decolourization. This might be due to the fact that as dye concentration (substrate) increases the active sites of the enzyme are saturated by the dye molecules so that after complete saturation there is no ability for the enzyme to attach more dye molecules which remain in solution thus decreasing % decolourization. In previous literature, the maximum colour removal (85.7 %) in case of decolourization of RBBR by soybean peroxidase was obtained at concentration of 40 ppm[24].

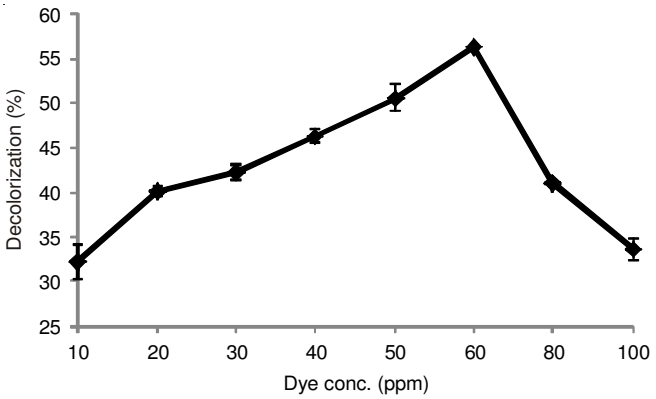

Fig. 4. Effect of the dye concentration on the decolorization of the drimarene orange KGL

Optimum temperature for decolourization of drimarene orange KGL: The enzyme was found to be thermostable as maximum decolourization of drimarene orange KGL was achieved at 60 °C, however a decrease in de-colourization was reported above and below this optimum (Fig. 5). The decrease in dye decolourization at higher temperature might be due to the denaturation of enzyme at higher temperature which resulted in low activity and hence low decolourization. Below the optimum temperature, the enzyme might not be able to achieve its energy of activation for reaction with the dyes, hence again low decolourization was observed. Consistent with our work, maximum degradation of Disperse Red 343 with horseradish peroxidase[25] and direct dyes (Solar Blue A and Solar Flavine 5G) by *Raphanus sativus* peroxidase has been observed to be around at 50 °C[23].

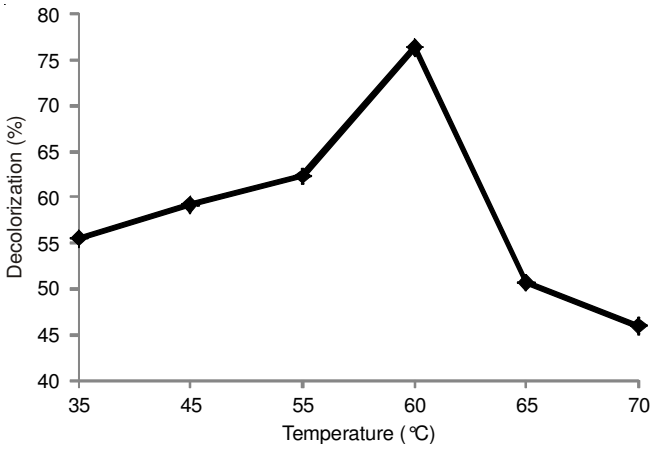

Fig. 5. Effect of the temperature on the decolorization of the drimarene orange KGL

Optimum time of contact for decolourization of drimarene orange KGL: The results regarding the decolourization of drimarene orange KGL as a function of contact time with the CFP are shown in Fig. 6. It is evident from the data that 160 min of the reaction time is sufficient for the maximum dye decolourization (81.36 %). In previous literature, eight reactive dyes were decolourized by bittergourd peroxidase out of which four dyes (RB 4, RB 171, RR 120, RB 160) were maximally decolourized within 60 min. However all the remaining took longer time duration for maximum decolourization. Actually, the reaction time is directly related to the different structures of the dyes; this fact affected the way of enzyme activity and consequently could cause variation in the reaction time[26].

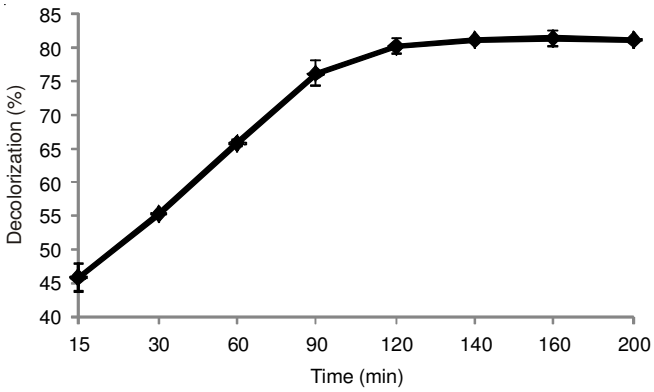

Fig. 6. Effect of the time of contact on the decolorization of the drimarene orange KGL

Conclusion

The present study revealed that peroxidase isolated from orange (*Citrus reticulata*) leaves has a high decolourization potential for drimarene orange KGL as 81.36 % decolourization was achieved by optimizing different parameters without utilizing any redox mediator.

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