Soybean Peroxidase Catalyzed Decoloration of Acid Azo Dyes

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Introduction

Synthetic dyes are composed of stable chemical compounds which are commonly released into wastewater and represent a serious water pollution issue in the textile industry.1

In recent decades, tremendous development in the industrial and agricultural fields has led to the use of chemical compounds that are released into the environment in the form of toxic contaminants.2 Dyes are among these compounds, used in many industries such as furniture manufacturing, textiles, food production and paint.3 Thousands of dyes were developed and used at the beginning of the 20th century.4 The presence of low concentrations of dyes in aqueous solutions can be toxic.5 In some situations, more than 15% of the transferable dyes are lost to surface water.6 Some compounds that are produced from partial decomposition of dyes, such as aniline, are carcinogenic, toxic and mutagenic.6 Some of the major causes of environmental risk are related to the recalcitrance of dyes to biodegradation.7 Two azo dyes were chosen for this study, Acid Black 2 and Acid Orange 7 (Figure 1 and Table 1). Acid Orange 7 is a simple monoazo dye, found in wastewater of cosmetic and textile industries.8 Acid Black 2 is a structurally more complex, bisazo dye used to make India ink.9 Various degradation procedures for dyes have been reported in the literature. Physical methods often used include sonophotolysis, semiconductor photo-catalysis, microwave methods, non-thermal plasma processing, photocatalytic-degradation and adsorption.10-14 Various chemical procedures have been employed, such as electro-chemical oxidation, reaction with coated and treated metal oxides, Fenton and photo-Fenton processes, and other advanced oxidation processes using different catalysts.15-22 Biological treatments have also been reported in the literature, such as bacterial decolorization, aerobic biological treatment, cyanobacteria...
reactivity, and fungal decolorization.\textsuperscript{1, 4, 21, 23, 24}

Incomplete color removal, cost and/or production of secondary pollutants are disadvantages of these methods.\textsuperscript{25} In addition, the generation of hazardous by-products, large amounts of sludge and low efficiency are problems associated with conventional treatment methods.\textsuperscript{27}

A new method based on the use of enzymes such as peroxidases was developed by Klibanov et al. in 1980.\textsuperscript{28} During the last decades, the application of enzymatic treatments underwent great development such as identifying new substrates and various peroxidases in addition to understanding mechanisms of reaction and inactivation, the effect of additives and different reactor configurations.\textsuperscript{29} Reaction under mild conditions and at high reaction rates are the main advantages of dye degradation using enzymes.

Some azo dyes have been decolorized by using peroxidases such as soybean peroxidase (SBP), lignin peroxidase, manganese peroxidase, and horseradish peroxidase, as well as laccases.\textsuperscript{30} It has been suggested that enzymatic treatment could oxidize the dye structures to form compounds with lower toxicity and may eventually mineralize the dyes through enzymatic radical formation, and subsequent removal.\textsuperscript{31}

Previous studies have examined chemical and enzymatic decoloration, but few have dealt with the use of inexpensive crude enzymes such as SBP in the decoloration of acid dyes. Only one recent report used the same SBP preparation as the present study, which highlights the need for continued exploration of the scope for enzymatic azo dye treatment in order to arrive at a generalized treatment process.\textsuperscript{25} Soybean seed coat peroxidase is well suited for this study because of its stability, ease of extraction, widespread availability and potential for adding to the soy value chain.\textsuperscript{33}

The present study evaluated the feasibility of SBP catalyzed oxidative dye polymerization and removal of Acid Orange 7 and Acid Black 2.

**Methods**

Crude dry solid SBP (Enzyme Commission number 1.11.1.7, industrial grade, lot number 18541NX, Reinheitszahl value of 0.75 ±0.10) was obtained from Organic Technologies.

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### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| $H_2O_2$     | Hydrogen peroxide |
| SBP          | Soybean peroxidase |

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**Figure 1 — Chemical structures of Acid Orange 7 and Acid Black 2 dyes**

![Chemical structures of Acid Orange 7 and Acid Black 2 dyes](image)

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**Table 1 — General Characteristics of Acid Orange 7 and Acid Black 2**

| Molecular formula | Color index | Molecular weight (g mol$^{-1}$) | Water solubility (g L$^{-1}$) |
|-------------------|-------------|---------------------------------|------------------------------|
| Acid Orange 7     | HOC$_6$H$_5$N=NCe$_3$H$_3$SO$_3$Na | 15510                           | 116                          |
| Acid Black 2      | C$_6$H$_5$N$_6$ | 50420                           | 50                           |

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(Coshcton, OH, USA). Dry solid bovine liver catalase (Enzyme Commission number 1.11.1.6, lot number 120H7060, 19900 U/mg solid) was purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA). Acid Black 2, i.e. Nigrosin water soluble, and Acid Orange 7, i.e. Orange II sodium salt, were purchased from Sigma-Aldrich Chemical Company. The enzymes were stored at -15°C, and their sub-stock solutions (70 U/mL for SBP and 17000 U/mL for catalase) were stored at -4°C. Hydrogen peroxide (30% wt/vol) was provided from ACP Chemicals, Inc. (Montreal, Quebec) and stored at 4°C. All other chemicals were of highly pure or analytical grade purchased from Sigma-Aldrich or BDH Chemical Corporation, Inc., (Toronto, Ontario). The spectrophotometer used was a Carey 100, Varian Australia.

**Soybean peroxidase activity assay**

A spectrophotometric procedure was used to measure free SBP activity. Briefly, this assay determines the rate of color formation at 510 nm due to the quinone imine formed in the oxidative coupling of phenol and 4-aminantipyrine catalyzed by SBP in the presence of hydrogen peroxide. The assay utilized a reagent consisting of 10 mM phenol, 40 mM phosphate buffer (pH 7.4), 2.4 mM 4-aminantipyrine, and 0.2 mM hydrogen peroxide (H₂O₂) in a total volume of 950 μL. The reaction was then started by adding 50 μL of the diluted enzyme solution to 0.95 mL of the reagent, and the initial rate of color formation at 510 nm was monitored for 30 seconds. The sample dilution was adjusted according to the expected enzyme activity (to have approximately 0.2 absorbance change in 30 seconds). One unit of SBP activity is defined as the amount catalyzing 1 μmol of hydrogen peroxide conversion per minute under the assay conditions.

**Dye assays**

Since the dyes absorb in visible wavelengths, all the samples were examined by direct spectrophotometric determination at 605 nm for Acid Black 2 and 510 nm for Acid Orange 7.

**Buffer preparation**

Buffer preparation was accomplished according to the method outlined by Stoll and Blanchard. Acetate buffer with a pH range of 3.6-5.6, phosphate buffer with a pH range of 5.7-8.0, and carbonate-bicarbonate buffer with a pH range of 9.2-10.7 were used to obtain the proper pH control.

**Experimental protocol**

Batch reactors were used for Acid Black 2 and Acid Orange 7 to remove 95% of the substrate. The parameters that were optimized were pH, enzyme activity and hydrogen peroxide concentration. All experiments were conducted at room temperature in 20-mL glass flasks with 0.5 mM substrate. The buffer used was acetate, carbonate or phosphate (40 mM) to cover the pH range of 3.5 to 8.0. The stirring time was 3 hours using a magnetic stirrer and Teflon-coated magnetic stir bars for all experiments at room temperature. The reaction time was chosen to be consistent with previous studies of 95% phenol and aniline degradation. The optimal conditions to remove 95% of these dyes were determined. This degree of removal was chosen because 5% remaining can be detected with high precision, while the range of 1-4% remaining would be close to or below the detection limit of the analytical procedure employed.

The components of the sample mixture were added at the appropriate concentrations in the following order to initiate the reaction: water, buffer, dye, SBP and hydrogen peroxide. Reactions were stopped by adding excess catalase to 17 U/mL final concentration to quench the residual hydrogen peroxide by quick consumption.

The samples were then centrifuged and micro-filtered using a 0.45-μm micro-syringe filter before spectrophotometric determination. All experiments were conducted in triplicate under the same conditions and the corresponding standard deviations were calculated and are represented by the error bars provided in Figures 2-5.

Time course studies, in 200 mL batch reactors, with 0.5 mM substrate (Acid Orange 7 or Acid Black 2), were carried out under the optimal conditions of pH, minimum hydrogen peroxide and enzyme concentrations. Aliquots (5 mL) were withdrawn by micropipette at various time intervals, quenched with 0.25 mL catalase solution and then centrifuged, micro-filtered and analyzed by a spectrophotometer. Centrifugation was used for quick sample preparation in order to get a clear liquid sample.

**Results**

Qualitatively, both dye reactions produced precipitates and the solutions were decolorized. The Acid Orange 7 solution was converted to a pale yellow from deep orange with small yellowish precipitate particles, while the Acid Black 2 solution was converted to a colorless one with larger blue precipitate particles. Centrifugation and microfiltration were conducted for all samples before spectrophotometric determination to remove any interference during measurement. Studies of the most important parameters, pH, enzyme activity, hydrogen peroxide concentration and time course were performed in completely-mixed batch reactors. Such information...
would allow the design of a treatment system and prototype reactor. Enzyme immobilization, often considered for enzymatic treatment systems, was not a viable option here because of the formation of solid products.

Optimization of dye decoloration from water

The experiments were designed to test the effect of pH under stringent conditions with respect to enzyme activity (insufficient activity to achieve complete conversion of substrate) to clearly accentuate the pH effect, as seen in Figure 2. The optimum pH for the enzymatic conversion of Acid Black 2 was in the acidic range (4.3-4.8), while the optimum pH for Acid Orange 7 covered a broad range of neutral conditions (6.0-8.0).

Enzyme activity

The successive increase in the activity of the SBP at the respective optimal pH values was studied over 3 hours (Figure 3). The goal of the experiments was to achieve 95% removal of dyes. As seen, equimolar hydrogen peroxide was insufficient to achieve 95% removal with the range of enzyme activity 0.1-1.5 U/mL (compare Figure 3 symbols ♦ with △ and □ with ×). Figure 3 shows that the minimum SBP activity required for 95% conversion of dyes was 1.2 U/mL, which was chosen as the minimum effective enzyme activity for subsequent experiments.

Optimum H$_2$O$_2$-to-substrate concentration ratio

Using previously established optimal enzyme activity and pH, molar concentrations of H$_2$O$_2$ in the range 0.25-1.75 mM were studied for the enzymatic reaction of 0.5 mM Acid Orange 7 and Acid Black 2 over 3 hours with the goal of 95% conversion (Figure 4). Chemical oxidation
(without enzyme) was minimal over the same peroxide concentration range. The minimum hydrogen peroxide/substrate molar ratio required to achieve 95% removal was 3.0 for Acid Orange 7 and 2.5 for Acid Black 2.

**Effect of reaction time**

The time courses for the enzymatic reactions of the dyes were determined at the respective optima for pH, enzyme activity and minimum hydrogen peroxide concentration. The progress curves, red and blue trend lines in Figure 5, show approximately 95% removal in 3 hours. For quantitative analysis, the data were fit to a pseudo-first-order model for both molecules, as indicated by the black lines drawn in Figure 5. The black solid lines represent the first 30 minutes and the black dashed lines signify the entire time. The data were fitted to: percentage remaining = (initial percentage) e^{-kt}, where the fitted value of the initial percentage should be close to 100% and k is the apparent first-order rate constant (Table 2).

Thus, fitting the entire time course (180 minutes) was only slightly less precise, giving lower rate constants by approximately 10% for Acid Orange 7 and 9% for Acid Black 2, respectively, with corresponding lower half-lives of 23.9 and 28.9 minutes.

**Discussion**

The current method is compared to other non-enzymatic peroxide-based methods for dye removal in Table 3 and compared to other enzymatic dye methods based on SBP in Table 4.

The optimum pH depends on the proper ionization of the catalytic residues of SBP during the enzymatic reaction and may also depend on the type of dyes and their ionization states as a function of pH. Soybean
peroxidase is active over a wide range of pH values (5.7–8.0). The pKₐs of the functional groups in the dyes (i.e. phenolic OH 9.9, anilinium NH 4.6, naphtholic OH 9.3) could play a role in the optimum pH, especially with Acid Black 2. Similar pH optima were reported in previous studies using SBP in the removal of Trypan Blue dye (pH 4.0), a bisazo dye, Acid Blue 113 (pH 4.0), a bisazo dye, and Direct Black 38 (pH 3.6), a trisazo dye. Soybean peroxidase enzyme with a pH range of 3–5 demonstrated decoloration of Crystal Ponceau 6R, a monoazo dye (but only in the presence of a mediator), methyl orange, a monoazo dye, and Remazol Turquoise Blue G 133, a phthalocyanine dye. In one study, a pH range of 4–6 was reported optimal using horseradish peroxidase in the decolorization of Naphthol Blue Black, a bisazo dye, and in another study, pH 2 was optimum in the decoloration of Direct Yellow 106, a complex monoazo dye by Cucurbita pepper peroxidase. The optima

| Method                          | pH     | [H₂O₂] mM/substrate] mM | Rate constant | Reference |
|--------------------------------|--------|-------------------------|---------------|-----------|
| **Acid Orange 7**              |        |                         |               |           |
| Fenton                         | 7      | 266.7/0.14              | k=1.5×10⁻² min⁻¹ | 38        |
| Ozone/H₂O₂                     | 6      | 0.23/0.227              | -             | 26        |
| Homogeneous Fenton              | 4      | 2/0.085                 | -             | 39        |
| Heterogeneous Fenton            | 3      | 7.77/0.227              | -             | 40        |
| Heterogeneous Fenton/ultrasonic | 3     | 4/0.028                 | k=(7 ± 0.001)×10⁻² min⁻¹ | 41        |
| Proposed method                 | 6.9    | 1.5/0.5                 | k=(3.2 ± 0.08)×10⁻² min⁻¹ |           |
| **Acid Black 2**               |        |                         |               |           |
| Solar Photo-Fenton              | 3-4    | 9.77/0.1                | -             | 42        |
| *UV-A Activated *ZnO/H₂O₂      | 8.17   | 9.77/4.06               | -             | 25        |
| Proposed method                 | 4      | 1.25/0.5                | k=(2.6 ± 0.1)×10⁻² min⁻¹ |           |

Abbreviations: k, rate constant; UV-A, ultraviolet; ZnO, zinc oxide

Table 3 — Comparison of Current Enzymatic Method with Other Non-Enzymatic Hydrogen Peroxide-Based Methods

Table 2 — Parameter Fit of Progress Curves for Color Removal of Acid Orange 7 and Acid Black 2

|                | k, min⁻¹   | Half-life, min | Initial percentage | R²     | Represented in Figure 5 |
|----------------|------------|----------------|-------------------|--------|-------------------------|
| **First 30 min** |            |                |                   |        |                         |
| Acid Orange 7   | (3.2 ± 0.1)×10⁻³ | 21.7           | 102 ± 1.1         | 0.9963 | Solid black line        |
| Acid Black 2    | (2.6 ± 0.1)×10⁻³ | 26.7           | 102 ± 1.7         | 0.9873 | Solid black line        |
| **Whole time**  |            |                |                   |        |                         |
| Acid Orange 7   | (2.9 ± 0.1)×10⁻³ | 23.9           | 99.4 ± 2.4        | 0.9892 | Dashed black line       |
| Acid Black 2    | (2.4 ± 0.1)×10⁻³ | 28.9           | 100 ± 2.3         | 0.9893 | Dashed black line       |

Abbreviations: min, minute; R², correlation coefficient; k, rate constant.
recommended pH for further work are 4.4 and 6.9 for Acid Black 2 and Acid Orange 7, respectively. A sharp reduction in the percent conversion of Acid Orange 7 was observed in the slightly acidic region, possibly due to a change in the ionization of the catalytic residue of the enzyme in such conditions.\textsuperscript{49} The active site of soybean peroxidase contains an imidazole group of the distal histidine residue, and the pK\textsubscript{a} of conjugate acid (protonated form) is 4.5.\textsuperscript{50} Soybean peroxidase requirements for degradation of some aromatic compounds were variable, as seen from previous studies: 0.6 U/mL for 1.0 mM aniline, 0.80 U/mL for 1.0 mM phenol, and 0.43 mU/mL for 0.10 mM benzidine.\textsuperscript{27,28,30} The same crude SBP as the present study required 0.75 and 1.5 U/mL for Acid Blue 113 and Direct Black 38, respectively, when prorated for the same dye concentration.\textsuperscript{32}

The increased hydrogen peroxide concentration requirement over that predicted theoretically for dimer formation (0.5 H\textsubscript{2}O\textsubscript{2}/dye) is consistent with further polymerization. Further oligomerization can occur, as long as the oligomers remain soluble and contain the requisite phenolic and/or anilino functional groups, until the solubility limit is reached, thus its precipitation.\textsuperscript{51} The additional cycles of oligomerization require additional peroxide. Thus, the extra H\textsubscript{2}O\textsubscript{2} consumed over the theoretical stoichiometric requirement is attributed to its consumption by the soluble dimeric and oligomeric compounds produced in the reaction. These results are consistent with earlier studies on the use of purified SBP in dye removal of Crystal Ponceau 6R.\textsuperscript{31} The same preparation of crude SBP required peroxide ratios of 2.5 and 5.0 for Acid Blue 113 and Direct Black 38, respectively.\textsuperscript{32}

The rate of reaction should be directly proportional to the enzyme concentration; thus, the reaction would be expected to progressively slow down with the loss of enzyme activity over the reaction period, with loss of activity caused by reactive radicals and/or end-product oligomers.\textsuperscript{37} This is evident in Figure 5 where the actual progress curves lag behind the fitted curves beyond 60 min.\textsuperscript{52}

### Conclusions

The present study found that crude SBP is a vital and effective catalyst for the decolorization and oxidative polymerization of hazardous aromatic azo dyes. The pH (Figure 2), hydrogen peroxide concentration (Figure 4), enzyme activity (Figure 3) and reaction time were key factors in the enzymatic treatment of the dyes. Limiting the amount of SBP resulted in lower substrate removal efficiencies, and excess peroxidase had no detrimental effect on the removal of the dyes (Figure 3). In addition, first-order fitting accounted reasonably well for the reaction progress curves (Figure 5).

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| Dye                        | SBP concentration | pH | [H\textsubscript{2}O\textsubscript{2}] mM/substrate mM | Removal % | Reference |
|----------------------------|-------------------|----|-----------------------------------------------|-----------|-----------|
| Acid Blue 113              | 1.5 U/mL          | 4  | 2.5/1.0                                       | 95        | 32        |
| Direct Black 38            | 0.75 U/mL         | 3.6| 2.5/0.5                                       | 97        | 32        |
| Remazol Turquoise Blue G 13 | 2.06×10\textsuperscript{-7} M | 3.3| 0.0998/0.18                                   | 96        | 43        |
| Methyl Orange              | 0.373 U/mL        | 5  | 2/0.09                                        | 81        | 44        |
| Crystal Ponceau 6R        | 0.27 µM           | 5  | 0.175/0.059                                   | 100       | 31        |
| Trypan Blue                | 40 U/mL           | 4  | 0.064/0.045                                   | 90        | 30        |

*Table 4 — Comparison of the Studied Dyes with Other Dyes Using the Soybean Peroxidase Decoloration Method*
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