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ENZYMATIC TREATMENT WITH SOYBEAN PEROXIDASE OF AN AZO-DYE, DIRECT BLACK 38, AND AN AZO-DYE PRECURSOR, 4-CHLORO-O-TOLUIDINE

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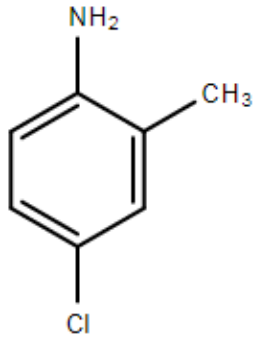
Abstract: Aromatic compounds such as anilines and dyes are present in industrial wastewater. Azo-dyes, such as Direct Black 38 (DB38), are the major group of dyestuffs used in the textile industry whereas 4-chloro-*o*-toluidine is an intermediate in production of Pigment Red 7. Both compounds are classified as probable human carcinogens by the U.S. EPA. This research focused on crude soybean peroxidase (SBP), which catalyzes the oxidative polymerization of hazardous pollutants in the presence of H_2O_2. The optimum operating conditions investigated were pH, H_2O_2 concentration and SBP activity (in U/mL; where U is a standard unit of catalytic activity) required to achieve at least 95% conversion. For 4-chloro-*o*-toluidine at 141 ppm, the optimal conditions to achieve 95% conversion were observed at an acidic pH in the range of 3.5-4.5, <50 mU/mL SBP and 0.5-0.6 mM H_2O_2. For DB38 at 391 ppm, the optimal conditions to achieve 95% color removal were pH 3.6, 3.0 U/mL SBP and 2.5 mM H_2O_2. To decrease the concentrations of SBP and H_2O_2 needed, a two-step process was also studied for the dye, in which zero-valent iron (Fe°) reduction was used before SBP treatment. Reduction of the azo-bond with Fe° produced colorless solutions; however the toxic products formed after Fe° reduction, such as aniline and benzidine, must be treated with SBP for their removal. Pre-treatment by Fe° followed by 0.6 U/mL SBP and 1.1 mM H_2O_2 at pH 5.0 were needed for more than 95% color, dye, aniline and benzidine removal.

Keywords: azo-dye, 4-chloro-*o*-toluidine, soybean peroxidase, Fe° reduction

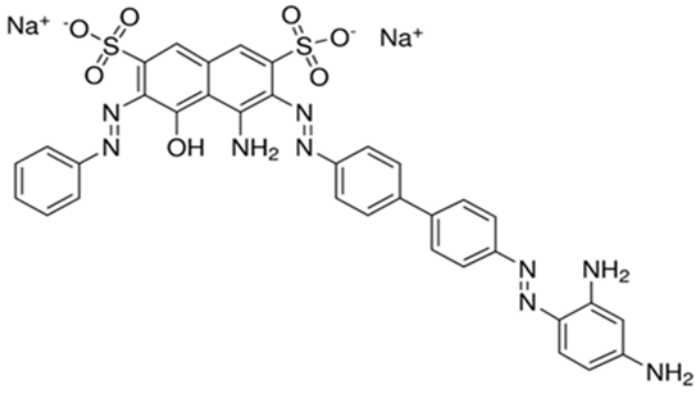
1 Introduction

Azo-dyes are the major group of dye-stuffs used in industries such as textile, printing, leather among others. The presence of color in water bodies reduces the photosynthetic capability of aquatic animals and plants. Around 12% of dyestuffs are released to the ecosystem (Ali *et al*., 2013). On the other hand, 4-chloro-*o*-toluidine is an organic compound which is used to produce azo-dyes *in situ* on nylon, cotton silk and acetate as well as an intermediate for pigments such as Pigment Red 7 and Pigment Yellow 49 (IARC, 2018). The compounds DB38 (which is a representative of the azo-dye group) and 4-chloro-*o*-toluidine, studied in this paper, are classified as probable human carcinogens by the U.S. EPA (Figure 1) (IARC, 2018). Benzidine-based dyes, such as DB38, are a challenge for treatment since no single treatment system is adequate and efficient to treat it due to its various sub-structures. Processes, such as AOPs (advanced oxidation processes), have been used to treat textile wastewater efficiently (Sudha *et al.,* 2014). Nadeem *et al*., (2017) used as a final step (polishing treatment) for real textile wastewater, a combination of $UV/H_2O_2/O_3$ as the most effective combination in terms of potential for water reuse with just over 600,000 m^3/yr, for total color removal and for 60% COD removal. However, AOP processes have several disadvantages such as high energy cost. Conventional techniques to remove azo-dyes have been reported such as chemical

oxidation, adsorption or coagulation and flocculation process. However, disadvantages such as high cost, sludge production or formation of toxic products create the need to have more environmentally friendly techniques (Bandala *et al.,* 2008).



4-chloro-*o*-toluidine



Direct black 38 (Chlorazol black)

Figure 1. Structures for compounds, 4-chloro-*o*-toluidine and DB38

1.1 Enzymatic treatment

During the early 1980s horseradish peroxidase (HRP) was used to treat aromatic compounds such as phenols and anilino compounds (Klibanov and Morris, 1981). Characteristics such as simplicity and ease of control as well as small footprint, enzyme specificity and the broad range of pH and temperature over which enzymes can work, are the advantages of enzymatic treatment compared to conventional processes. Advances in the industry to make the application of enzyme a practical alternative are needed in order to overcome the disadvantage of enzyme cost (Steevensz *et al*., 2014; Karam and Nicell, 1997). This treatment can be used as a primary or polishing treatment in conjunction with a biological process (Steevensz *et al.,* 2014; Nadeem *et al*., 2017).

Peroxidases are hemoproteins which catalyze the oxidation of an extensive range of substrates in the presence of H_2O_2 (Gijzen *et al*., 1993). The soybean seed coats (hulls) are a by-product from crushing operations that are used as animal feed. Washing these hulls with water, extracts SBP without compromising the feed value of the hulls. The activity of the SBP varies among the cultivars and seed coats (Steevensz *et al.,* 2013).

1.2 Zero-valent iron reduction process

Azo-dyes are normally resistant and difficult to degrade. For this reason a pre-treatment is recommended. Azo linkages are easily reduced to produce colorless solutions which contain amines that can be treated later (Sudha *et al.,* 2014). Fe° is an environmentally friendly reducing agent which produces a colorless solution when reacting with azo-dyes to reduce the azo-bond. This azo cleavage results in products which require further treatment (Fan *et al.,* 2009). Reductive cleavage of the azo-bond with Fe° might be a step-wise reaction (Equation 1 and 2). First, two electrons will be transferred from Fe° to transform the azo-dye into a hydrazine-intermediate, which will be further reduced with two more electrons, generating the substituted aniline (Gooding *et al.,* 1996; Nam, 2000; Weber, 1996).

[1] $R_1 - N = N - R_2 + 2H^+ + 2\bar{e} \rightarrow R_1 - NH - NH - R_2$

[2] $R_1 - NH - NH - R_2 + 2H^+ + 2\bar{e} \rightarrow R_1 - NH_2 + R_2 - NH_2$

2 Materials and Methods

2.1 Materials

Crude SBP was obtained from Organic Technologies (E.C. 1.11.7, Industrial Grade lot #18541NX, RZ = 0.750 activity 5 U/mg) (Coshocton, OH). Catalase from bovine liver (CAS 9001-05-2, lot #SLBB1797V, activity 2000-5000 U/mg protein) was purchased from Sigma Aldrich Chemical Company Inc. (Oakville, ON). The solutions of enzyme and catalase were stored at 4°C. Hydrogen peroxide (H_2O_2, 30% w/v) was purchased from ACP Chemicals Inc. (Montreal, PQ). Direct Black 38 (DB38, >45% purity) and 4-chloro-*o*-toluidine (98% purity) were purchased from Sigma-Aldrich Chemical Company Inc. DB38 was used without any further purification. Iron filings, 40-60 mesh, MSDS IX0210 were purchased from Innovating Science through Fisher Scientific, New Jersey.

2.2 Methods

Color reduction was analyzed with a UV-VIS spectrophotometer at the λmax of DB38 (520 nm).

In the present investigation, DB38 was treated in a single-step process with SBP in the presence of hydrogen peroxide (H_2O_2), and also in a two-step process using Fe° reduction followed by enzymatic treatment. First, for single-step process parameters optimized for maximum removal were: H_2O_2 concentration (mM), pH and enzyme concentration (U/mL). For enzymatic treatment, batch reactors with 0.5 mM DB38 (391 ppm) were treated using different pH and concentrations of H_2O_2 and SBP during 3 h reaction. Optimization was done one parameter at the time. For the two-step process, iron fillings have to be washed with 10% HCl to obtain higher surface area and then were washed with 15 mM carbonate buffer and 1 mM sodium sulfite to remove the metal oxides and chlorides. At the end fillings were washed with 20 mM sodium sulfite (with 0.1% cobalt chloride with respect to sodium sulfate) to remove alkalinity and create anaerobic conditions and stored that way at room temperature. For the two-step process, the Fe° reduction was tested for different reaction times and amounts of iron (1.0, 1.5, 2.0 and 2.5 g) in the presence of sodium sulfite (with 1%w/w cobalt chloride) for maximum color removal (0.5 mM DB38 initial concentration) in a 40 mL batch reactor. Then, the solution was filtered and subjected to enzymatic treatment where pH, SBP and H_2O_2 were optimized for maximum removal of sub-products, aniline and benzidine. The latter were formed after Fe° as a result of the azo-dye reduction. For aniline, benzidine and 4-chloro-*o*-toluidine an HPLC Model 2487 dual wavelength absorbance detector, Model 1525 binary HPLC pump and Model 717 auto sampler operated by Breeze 3.3 software was used. For aniline and benzidine, determination was done with 1 mL/min flow, 70% of 5 mM ammonium acetate and 30% acetronitrile at 25°C monitored at 280 nm.

4-chloro-*o*-toluidine was treated using only single-enzymatic treatment. Removal of 4-chloro-*o*-toluidine was monitored by HPLC. pH, enzyme and H_2O_2 concentrations were optimized after 3 hour reaction for a starting concentration of 1mM 4-chloro-*o*-toluidine (141 ppm). The optimization process was varying one parameter at the time, as done for DB38. HPLC methodology was 60% acetronitrile and 40% ammonium acetate (5 mM) 30°C at 1 mL/min monitored at 290 nm.

3 Results

3.1 pH optimization

Enzymatic treatment has proven to be pH dependent (Kalsom *et al.,* 2013, Mazloum *et al*., 2014; Chiong *et al.,* 2016). The optimum pH for treating 141 ppm 4-chloro-*o*-toluidine was in the acidic range, 3.5-4.5. There was reduction in the conversion of the substrate at more basic pH, which could be due to a change in ionization of catalytic residues of the enzyme. The optimum pH depends on the ionisation of the catalytic residues of SBP during the enzymatic reaction as well as the type of the aromatic substrate (Mousa Al-Ansari *et al.,* 2009). A similar result was seen previously for 0.1 mM benzidine and 1 mM aniline using SBP where the optimum was pH 5, for both compounds (Altahir *et al.,* 2015; Mazloum, 2014).

For the single-step process, the optimum pH for DB38 was 3.6. Other authors have also found the optimum pH for azo-dyes to be in the acidic range using different peroxidases (Ali *et al.,* 2013; Kalsoom *et al.,* 2013; Kokol *et al.,* 2007). Ali *et al*. (2013) found that for 40 mg/L of azo dye Crystal Ponceau 6R (CP6R) with SBP optimal pH was in the range 3 to 5.

3.2 H_2O_2 optimization

H_2O_2 is a co-substrate that initiates the enzyme reaction mechanism. Excess or low concentration of H_2O_2 can affect the enzymatic reaction by acting as an inhibitor or limiting factor during the enzymatic reaction (Dunford, 1999). Different concentrations of H_2O_2 were tested at optimal pH to obtain up to 95% removal of both compounds. For 4-chloro-*o*-toluidine (1mM) the optimum H_2O_2 was found in the range of 0.5-0.6 mM. This ratio is in agreement with the expected theoretical value, where from each mole of H_2O_2 consumed two moles of aromatic compounds are converted to the free radicals. These free radicals then form dimers and through subsequent enzyme cycles form larger oligomers and precipitate out of solution when the solubility limit is reached (Yu *et al.,* 1994).

To have more than 95% color removal for 0.5 mM DB38, 2.5 mM H_2O_2 was required at optimal pH 3.6. Chiong *et al.,* (2016), for methyl orange dye at 30 mg/L (0.09 mM), found an optimum value of 2.0 mM H_2O_2 that achieved around 80% decoloration. Kalsoom *et al.,* (2013) recommended a stepwise addition of H_2O_2 to obtain higher removal, for example with a total time of 15 minutes and addition of H_2O_2 every 3 minutes more than 90% color removal was achieved for 10-40 mg/L Trypan Blue (0.011 mM- 0.046 mM). The higher requirement of H_2O_2 for DB38 can be due to the impurities of the dye (this dye was >45% purity and used in all testes without further purification).

3.3 Enzyme optimization

With the optimal pH and H_2O_2 concentration found above, different concentrations of enzyme were tested to obtain up to 95% removal. For 4-chloro-*o*-toluidine the minimum SBP concentration required to achieve at least 95% conversion was <50 mU/mL. Similar amount of enzyme requirement was observed to obtain 95% removal with benzidine as a substrate for which 0.43 mU/ml SBP was required (Altahir *et al.,* 2015).

By contrast, under optimal concentration of H_2O_2 and pH, for more than 95% color removal of DB38 (0.5mM) 3 U/mL SBP was required; due to the high concentration of enzyme needed for DB38, a two-step process was studied. The purpose of the two-step process was to reduce the concentration of H_2O_2 and SBP needed using an environmentally friendly and relative cheap pretreatment, Fe° reduction.

3.4 Two-step process for DB38

First a Fe° reduction was done in an attempt to achieve the maximum color removal. The breaking of the azo-linkage results in the formation of 4 aromatic amines from DB38. Using aniline and benzidine as representatives of these four, an enzymatic treatment was studied to degrade this sub-products recovered after the Fe° reduction.

After zero-valent iron treatment, two representative products from azo-splitting, aniline and benzidine were quantified. Afterwards, pH, H_2O_2 and enzyme concentration were optimized for the enzymatic step to achieve more than 95% color, aniline and benzidine removal.

For the two-step process 2 g Fe° (for 40 mL of 0.5 mM dye) and 120 minutes' reaction time followed by 0.6 U/mL SBP and 1.1 mM H_2O_2 at pH 5.0 were required for greater than 95% color reduction as well as aniline/benzidine removal. Pure aniline and benzidine have been proven to be good enzyme substrates (Mazloum, 2014; Altahir *et al.,* 2015). Aniline optimal conditions were pH 5, 0.6 U/mL and 1.5:1 ratio peroxide to substrate (Mazloum, 2014). Meanwhile, benzidine requirements are lower, for 0.10 mM benzidine the optimal pH was 5. The SBP concentration was 0.43 mU/mL, and peroxide to substrate ratio of 1.5:1 (Altahir *et al.,* 2015). The results from this paper with DB38 after iron reduction agree with the presence of aniline/benzidine after Fe° with a change in optimal pH (pH 5) compared to the single-step process (pH 3.6), as well as the reduction in SBP and H_2O_2 concentrations.

4 Conclusions

Enzymatic treatment is an effective method to treat azo-dye and 4-chloro-*o*-toluidine. SBP effectively decolorizes more than 95% DB38 in the presence of H_2O_2 but at relatively high SBP and H_2O_2

concentrations. Pre-treatment of the dye using Fe° followed by enzymatic treatment allowed 5-fold reduction in the SBP required and 2-fold reduction in the H_2O_2 needed to decolorize more than 95% of the substrate while removing products formed during Fe° reduction of DB38.

SBP treatment of 4-choro-*o*-toluidine was very efficient achieving more than 95% removal with less than 50 mU/mL SBP and 0.5-0.6 mM H_2O_2.

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