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Rapid decolorization of anthraquinone and triphenylmethane dye using chloroperoxidase: Catalytic mechanism, analysis of products and degradation route

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HIGHLIGHTS

- Very efficient enzymatic decolorization for anthraquinone and triphenylmethane dye.
- Products analysis indicated it is a good alternative for biodegradation of these compounds.
- No restriction for dye species due to the active oxidants generated online in catalytic cycle.
- Strong toleration to high saline wastewater was observed ensuring it potential application.

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ABSTRACT

A rapid and efficient enzymatic decolorization of anthraquinone (Alizarin Red) and triphenylmethane dyes (Crystal Violet) by a heme peroxidase, chloroperoxidase (CPO), was presented in this work. The decolorization efficiency of Alizarin Red reached 98.23%, and that of Crystal Violet was 97.68% both within 7 min at mild condition with an enzyme concentration below ppm level. A characteristic mechanism was involved in this enzymatic oxidation, in which some small intermediates with high oxidative activity generated online during enzymatic catalytic cycle. The decolorization was in fact carried out in bulk solution instead of in heme, which can get rid of the restriction for the size of dyes required by the channel access to enzymatic active site. UV–vis and HPLC–MS analysis of products indicated the chromophoric groups were destructed and the dye molecules were broken-down into small pieces, indicating an increase in biodegradability of the dyes. COD and TOC values of dyes solution were all decreased. Meanwhile, the strong toleration of this CPO-catalyzed oxidative decolorization to the typical salt species (NaCl, $NaNO_3$, and Na_2SO_4) was observed, which showed a potential application of this method in treatment of industrial wastewater.

1. Introduction

Though no recent data is available on worldwide dye production, annual production of over 700,000 tones has been often reported in the literature [1–3]. In the textile industry, up to 50% of the dyes are lost after dyeing process and about 10–15% of them are discharged in the effluents [4]. Color is usually the first contaminant in these effluents. A very small amount of dye in water (10–20 mg L^{-1}) is highly visible. These dyes in water can affect water transparency and sunlight penetration [5,6]. Release of these dyes into the environment has become a major concern not only because these brightly colored, water-soluble dyes are particularly problematic for color pollution in water bodies [7,8], but also because their metabolites may be mutagens or carcinogens.

These synthetic dyes have stable chemical structure to meet various coloring requirements. Based on the chemical structure of the chromophoric group, dyes are classified as azo, anthraquinone, triphenylmethane, arylmethane, acridine, heterocyclic, polymeric dyes, cyanine, or phthalocyanine *etc.*; Although azo dyes represent approximately 60–70% of all dyes used by the textile

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industry, anthraquinone dyes constitute the second largest class of textile dyes after azo dyes. They have chromophore groups, =C=O and =C=C=, and can be precipitated or adsorbed only in small amounts [9,10]. Following azo and anthraquinone dyes, triphenylmethane dyes (TPMs) are classed as the third largest class of dyes as far as dye consumption is concerned. TPMs dyes cause environmental concern because of their color and potential toxicity to animals and humans. For example, Crystal Violet is toxic to mammalian cells and is also a mutagen and mitotic poison [11]. Most countries have nominated triphenylmethane dyes as hazardous material and prohibited the use of them in aquaculture and food industry. However, they are still used in some areas due to their relatively low cost, ready availability and efficacy.

Considering the increasingly strict legislations and regulations, the associated industries are required to find economically viable and low-cost treatment of industrial dye-laden effluents before they are discharged into the environment. However, most dyes are known for their stability to light, heat and difficulty to be biodegraded. In treatment by activated sludge, over ninety percent of textile dyes entering activated sludge sewage plants pass through unchanged. Moreover, the inhibition of microbial growth by certain toxic dyes has been reported. Supplying a pre-treatment process of such wastewaters is therefore essential, but difficult. Most of the chemical and physical methods proposed for treating dye wastewater, such as ozonation, photooxidation, adsorption, ion exchange, membrane filtration and flocculation have technical and economical limitations because of the high cost, inefficiency for some soluble dyes, producing large amount of sludge, and resulting in higher pollution potential than the effluents because of the excessive use of chemicals [12,13].

Nowadays, the microbial-based biological treatment systems are getting more and more attention. Microbe is capable of decolorizing or degrading these recalcitrant compounds by mineralization of the target compounds. As regarding anthraquinone and triphenylmethane dyes, many microorganisms capable of decolorizing the two dyes have been reported over the past decade [14–16]. However, the effectiveness of these treatment systems depends upon the survival and adaptability of microorganisms during the treatment processes. Moreover, the reduction involved in the degradation sometimes can produce undesired anilines. Nowadays, enzymatic oxidative treatment has become an attractive alternative as enzymes provide more simple systems than a whole organism.

Since the main emphasis is focused on decolorization of the solutions, reports on the analysis of products are very scanty. Several degradation routes of azo dyes have been proposed [17–20], but little information on products from decolorization of triphenylmethane and anthraquinone dye is available [21,22].

The present study focused on decolorizing anthraquinone (Alizarin Red) and triphenylmethane dyes (Crystal Violet) using chloroperoxidase–H_2O_2 system in mild condition in the presence of some typical salt species (NaCl, $NaNO_3$, and Na_2SO_4) which usually coexists in dye wastewater. Chloroperoxidase (CPO) is a glycohemoprotein secreted by the fungus *Caldariomyces fumago*. It is now considered to be the most versatile heme containing enzyme. CPO exhibits peroxidase, catalase and cytochrome P450-like activities in addition to catalyzing the chlorination of activated C—H bonds. This enzyme has been applied to the decolorization of azo dyes in our laboratory previously [23].

High performance liquid chromatography–mass spectrometry (HPLC–MS) technique was employed to identify the major products of Alizarin Red and Crystal Violet. The possible degradation route involved in the process is discussed accordingly. COD and TOC removal efficiency were determined. The toleration of this method to the inorganic salt species generally in industrial effluents was also evaluated.

2. Materials and methods

2.1. Cultivation of Caldariomyces fumago and purification of CPO

C. fumago was cultured according to the method established by Morris and Hager [24]. Chloroperoxidase was isolated from the growth medium of the fungus using acetone rather than ethanol in the solvent fractionation step. Then, CPO was further purified by DEAE-Sephadex A-50 ion exchange column chromatography. The enzyme solution was concentrated to 12.4 mg ml^{-1} CPO with R_z 1.16 (R_z = purity standard = A_{398}/A_{280} = 1.40 for pure enzyme) and activity of 7928 U ml^{-1} based on the standard monochlorodimedon (MCD) assay [25].

2.2. Dyes and chemicals

Alizarin Red and Crystal Violet were from Sigma–Aldrich (The structures are shown in Fig. 1). Monochlorodimedon (MCD) was obtained from Fluka. All the other chemical regents, such as dipotassium hydrogen phosphate, potassium dihydrogen phosphate, hydrogen peroxide (30% in aqueous solution) were obtained from Xi′an Chemical Co., Ltd. These chemicals are of analytical grade unless otherwise indicated.

0.1 mol L^{-1} potassium phosphate buffer was prepared by mixing appropriate volume of 1.0 mol L^{-1} KH_2PO_4 and K_2HPO_4 stock solutions and then diluting. This buffer solution was adjusted to various acidic pH by 1.0 mol L^{-1} HCl.

2.3. Decolorization of Alizarin Red and Crystal Violet

Decolorization was carried out in 0.1 mol L^{-1} phosphate buffer in a centrifugal tube with total volume of 1.0 ml containing CPO (0–0.50 μmol L^{-1} for Alizarin Red and 0–0.15 μmol L^{-1} for Crystal Violet decolorization respectively), and dyes (0.10–1.00 mmol L^{-1} of Alizarin Red and 0.05–0.20 mmol L^{-1} of Crystal Violet respectively) with pH 2–4 at 20–80 °C during 30 min. The reaction was started by adding H_2O_2 (0–10.00 mmol L^{-1}). The effect of operating parameters (pH, concentration of enzyme, H_2O_2 concentration, initial concentration of dyes, reaction time and temperature) on decolorization efficiency were investigated by changing one factor at a time while the other parameters were kept constant.

Decolorization efficiency was determined by monitoring the characteristic absorbance (λ_{max}) of dyes at UV–Vis spectrophotometer (Shimadzu UV-1700), with Alizarin Red at 420 nm and Crystal Violet at 584 nm respectively:

$$\text{Decolorization efficiency } (\%) = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

The data reported were mean of three measurements, and presented with error bar.

2.4. Degradation products analysis

Samples were collected after 30 min reaction. The supernatant of crude solution of Alizarin Red was extracted 3 times by ethyl acetate, and that of Crystal Violet was extracted by dichloroethane. The combined organic extract was purified by rotary evaporation (0.09 MPa, 40 °C) to remove the solvent, and then was dissolved in methanol for HPLC–MS analysis.

An Esquire LC – ion trap mass spectrometer (Bruker Daltonics, Germany) equipped with an orthogonal geometry Electrospray Ionization (ESI) source was employed to determine the products of enzymatic decolorization. Nitrogen was used as the drying (8 L min^{-1}) and nebulizing (0.8 Bar) gas at 180 °C. Scanning was performed from m/z 100 to 1000 in the standard resolution mode.