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Decolorization of Naphthol Blue Black using the Horseradish Peroxidase

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Abstract This study evaluates the potential of the enzyme horseradish peroxidase in the decolorization of one common industrial azo dye, naphthol blue black. Studies are carried out to understand the process parameters such as pH, temperature and reaction time. The enzymatic decolorization of the dye was examined by UV-Vis spectrophotometer and LC-MS measurements. Temperature and pH conditions were optimized for obtaining high azo-dye decolorization. Azo-dye removal at a pH range 4-6 was found to be the highest for all temperatures. After 5 minutes of treatment, the color removal of dye was ca. 80-90%. The LC-MS and spectrophotometric analyses indicated that the decolorization of the azo dye with enzyme was due to the reduction of the azo bonds. This study verifies the viability of the use of the horseradish peroxidase in the decolorization of naphthol blue black.

Keywords Dye decolorization · Horseradish peroxidase · Naphthol blue black · Spectrophotometer · LS-MS

Introduction

The treatment of industrial effluents is a challenging topic in environmental science, as control of water pollution has become of increasing importance in recent years [1]. Water pollution due to discharge of coloured effluents from textile dye manufacturing and textile dyeing mills is one of the major environmental concerns in today's world. Strong colour

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imparted by dyes to the receiving aquatic ecosystems poses aesthetic problems as well as serious ecological problems, such as carcinogenicity and inhibition of benthic photosynthesis. Therefore, a number of techniques aimed at preferential removal of dyes from wastewater have been developed [2–5]. Azo dyes have been widely used as colourants in a variety of products such as textiles, paper and leather. Approximately half of all known dyes are azo dyes, making them the largest group of synthetic colourants [6–8]. These chemicals present a potential human health risk as some of them have been shown to be carcinogenic. Various chemical and physical methods, such as chemical coagulation and adsorption on activated carbon, are being used. However, these traditional methods mainly transfer the contaminants from wastewater to solid wastes, which may lead to a new kind of pollution [2, 9].

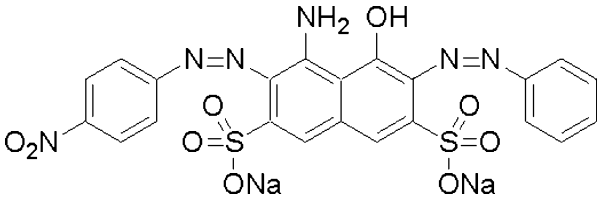
The use of enzymes is currently a possibility for application in environmental engineering, however their purification procedures are too expensive. Enzymes from various sources (fungus and plant based) have been applied for the treatment of dye based compounds [9]. The source of the selected enzyme and its nature along with system conditions have been found to have significant influence on its overall performance for pollutant removal. Fungal extracted enzymes have been mostly studied in dye removal processes[10–12]. Whereas, plant based peroxidases in the removal of pollutants have been less documented [13–16]. It has recently been demonstrated that extracellular enzymes of white rot fungi such as peroxidases (lignin peroxidase (LiP), horseradish peroxidase (HRP) and manganese peroxidase (MnP)) and phenoloxidase (laccase) can be used to degrade and detoxify polyaromatic hydrocarbons, polychlorinated biphenyls and certain dyes [17].

Peroxidases seem to be superior oxidizers, catalyzing decolorization of synthetic dyes; however, the peroxidative treatment is limited to water-soluble or -dispersible dyes. For the decolorization of water-insoluble dyes such as Solvent Yellow and Solvent Blue, a nonaqueous system has been thought to be advantageous, since organic solvents are required to dissolve them [18]. Specifically, the use of plant peroxidases in removal of phenolic pollutants from aqueous solution is well documented [19, 20]. It has been demonstrated that horseradish peroxidase (HRP) can catalyze free-radical formation, followed by spontaneous polymerization of a variety of aromatic compounds, including phenol [21–24], chlorophenols [13, 25] and other substituted phenols [26], in the presence of hydrogen peroxide (H_2O_2). Furthermore, HRP is also known to have the ability to degrade aromatic azo compounds in the presence of H_2O_2 and to precipitate industrially important azo dyes [15, 16, 27, 28]. Previous studies have shown that various phenolic and azo compounds have been degraded by HRP–H_2O_2 system [29].

Due to its high degree of fastness to light, the commercial grades of naphthol blue black (NBB) are widely used in the textile industry for dyeing wool, nylon, silk and textile printing. Other industrial use includes coloring of soaps, anodized aluminum and casein, wood stains and writing ink preparation. It has a structure consisting of azo, phenolic, anilino, naphthalene and sulfonated groups (see Scheme 1.). NBB is an industrially important acidic diazo dye, which has a high photo- and thermal- stability. Conventional methods of oxidative degradation, such as ozonation, usually do not effectively degrade these dyes. There is a need to find a better, more effective method for NBB degradation [30].

In this study, the decolorization of textile diazo dye NBB by the HRP was investigated. The influence of pH and temperature on enzymatic colour removal were determined. To our knowledge, naphthol blue black has not been investigated for degradation by HRP before. We found that the azo dye was decolorized with the use of HRP, and we then characterized the decolorization.

Scheme 1 Molecular structure of naphthol blue black



Experimental

Reagents

Horseradish Peroxidase (E.C. 1.11.1.7) (Mw ~ 40.000 Da) and Naphthol Blue Black (Mw: 616.50 g mol^{-1}) were purchased from Fluka. All chemicals that were used in the experiments were of analytical reagent grade and used as received without further purification. Ultra pure water was obtained from Millipore MilliQ system.

Measurement of Enzyme Activity

HRP activity was assayed by monitoring the oxidation of o-dianisidine at 30 °C and 460 nm [31]. One unit (U) of enzyme activity was defined as I μmol of oxidative compound produced in 1 min. All enzyme assays were carried out in a UV-1700 Pharmaspec Shimadzu Spectrophotometer.

Purification

The protein content of the purchased HRP (Fluka) was determined as 50% using the Bradford method [32]. HPLC (Viscotek GPCmax VE2001 GPC Solvent/sample module) chromatograms showed that there were impurities in purchased HRP (Fluka) enzyme. It was decided that using purified enzyme would be better for this study. Purchased HRP was purified by Affinity Chromatography using Concanavalin A-Sepharose 4B (con A-Sepharose 4B) as column material. In order to remove all the unbound impurities, the column was washed with 0.1 M acetate buffer, pH 6 containing 0.1 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. To elute the enzyme which was bound to the column material, the column was washed with 0.1 M acetate buffer, pH 6 containing 0.1 M methyl-α-D-mannopyranoside. The fractions containing HRP activity were collected and concentrated in ultrafiltration cell with Regenerated Cellulose membrane (Dia 25 mm, Mw 10.000) by washing 2 times with distilled water and 2 times with 0.01 M phosphate buffer, pH 7. RZ value (A_{460}/A_{280}) of the enzyme was determined as 2.24 [31]. The analytical determination was carried out in duplicate, and the average was used as a datumpoint. The activity of the free purified enzyme was 1781 U mg^{-1} at pH 7, 30 °C.

Azo Dye Decolorization by Horseradish Peroxidase

The decolorization was carried out directly in the spectrophotometer cuvette. The reaction was started by adding buffer solution at different pHs (50 mM asetate buffer in the pH range 3-5 and 50 mM phosphate buffer in the pH range 6-8), 12 μl dye (0.05 mM stock solution), purified HRP solution (5.88 U mL^{-1}) and finally 10 μl H_2O_2 (3%) as the initiator in the reaction cuvette respectively. The total volume was 3 mL. Dye decolorization was

measured spectrophotometrically with a UV-Vis. spectrophotometer (Model UV-1700 Pharmaspec Shimadzu) based on the maximum absorbance at 620 nm in the visible range, at different pHs (3,4,5,6,7,8) and temparatures (25, 30, 35,40, 45, 50, 60, 70 °C) for 60 minutes. Experiments were performed in triplicate and results were given as the mean values. The efficiency of color removal was expressed as the percentage ratio of the decolorized dye concentration to that of initial one.

$$\text{Dye decolorization}\,(\%) = \frac{A(i) - A(a)}{A(i)} \times 100$$

A(i) initial dye absorbance at 620 nm
A(a) dye absorbance after incubation at 620 nm

Kinetic Studies

The kinetic experiments were performed by varying the concentration of substrate (0,0125-0,05 mM) using constant enzyme and H_2O_2 concentration under the optimum conditions (at pH 5, 30 °C). The initial rates of decolorization and Michaelis-Menten constants (K_m) of purified HRP were determined by linear regression and the Lineweaver – Burk plots.

LC-MS Conditions

Analyses were performed with an HPLC-UV-ESI-MS system (Shimadzu LC-MS 2010 EV) with a C-18 column (20x0.21 cm, 5 μm) from Teknokroma. Water (0.1 mM ammonium acetate, pH 5.0) and acetonitrile (0.1 mM ammonium acetate) were used as mobile phases A and B

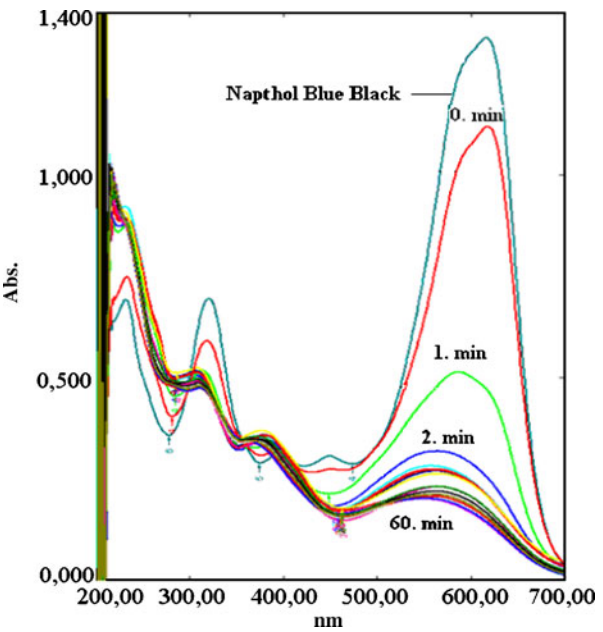


Fig. 1 UV-vis spectra recorded during the purified HRP-catalyzed decolorization of dye. Experimental conditions were 30 °C, pH 5.0 and total time 60 min. Each data point represents the average value of three independent experiments

respectively. Elution was adjusted in a gradient from 5% to 95% of B in 30 min. and flow rate was 0.2 mL min^{-1}. The mass spectra were acquired in negative ion mode over the mass range of 200 to 700 Da. Nebulising gas was nitrogen with a flow rate of 1.5 L min^{-1}. ESI probe tip voltage was set at -5 kV. Temperatures of heat block and curved desolvation line were set to 300 °C.

Results and Discussion

Enzymatic Decoloration of NBB

The obvious changes of enzymatic decoloration of dye using UV-vis absorbance spectra are shown in Fig. 1. In general, the absorbance at 400-700 nm corresponds to the n/p*

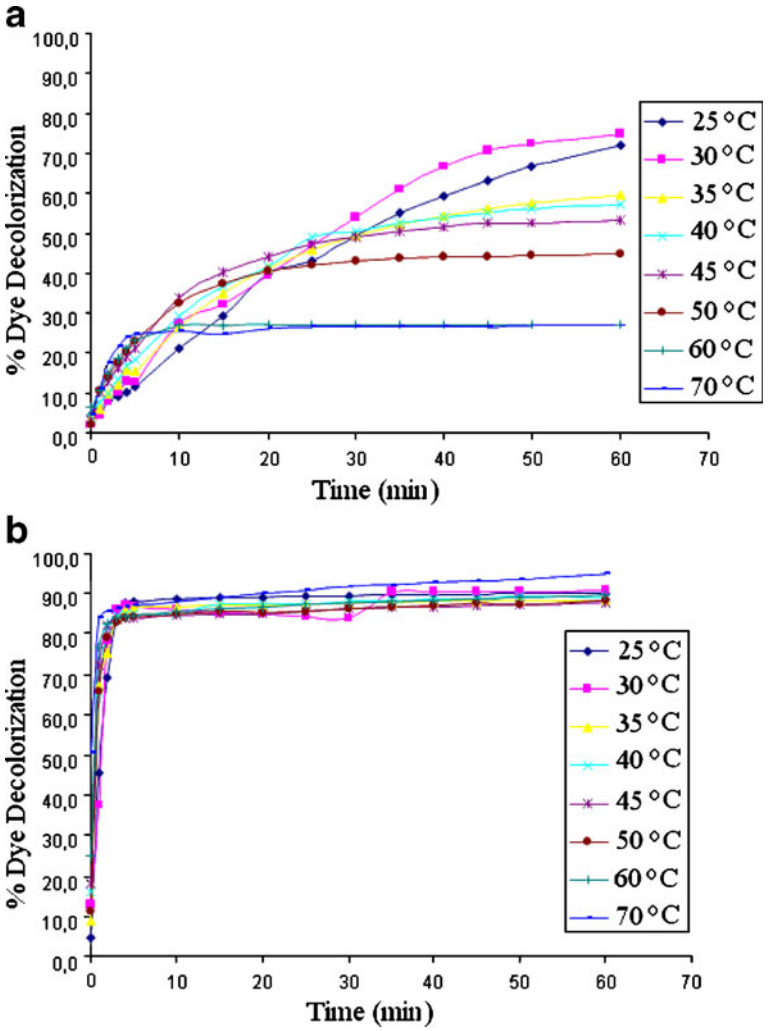


Fig. 2 Effect of temperature on decolorization of NBB by HRP: (**a**) at pH 7, (**b**) at pH 5. Conditions: dye concentration, 0.6 mmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 5.88 U ml^{-1}. Each data point represents the average value of three independent experiments

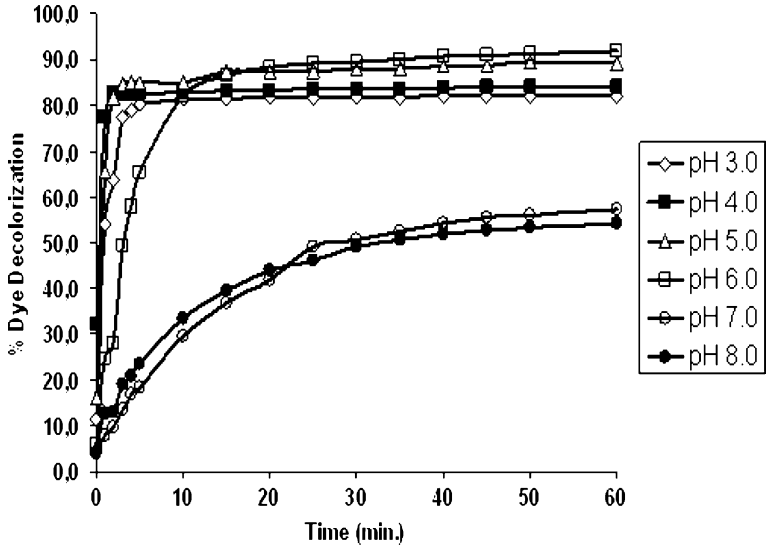


Fig. 3 Effect of pH on decolorization of NBB by HRP. Conditions: temperature, 40 °C; minute dye concentration, 0.6 mmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 5.88 U ml^{-1}. Each data point represents the average value of three independent experiments

transition of the azo and hydrazone forms, which is the origin of the color of azo dyes and is used to monitor the decoloration. The absorbance at 200-400 nm was attributed to the n/p* transitions in benzene and naphthalene rings of azo dyes. The decrease in this absorbance indicates the degradation of the aromatic part of the dye. These results are in agreement with the results reported in case of photocatalytic decoloration and degradation of azo dyes [33].

Figure 1 shows that both the absorbance at 200-400 nm and 400-800 nm of the dye decrease gradually with prolonging exposure time due to the increase in decoloration and degradation of the dye. These data clearly indicate that decolorization was very fast ca. 80-90% in the first 5 minutes of incubation period, then the rate of decolorization did not change significantly. It may be concluded that the enzymatic reaction of HRP is quite rapid, with most of the color removed in the first 5 minutes of contact with the enzyme.

Table 1 Color removal (%) of NBB by HRP at different temperature and pH after 60 minutes of treatment. Each value is a mean of duplicate experiments. Conditions: dye concentration, 0.6 mmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 5.88 U ml^{-1}

pH	Temperature (ºC)							
	25	30	35	40	45	50	60	70
3	79.5	82.1	83.4	82.0	81.8	59.3	39.1	35.4
4	83.5	83.3	81.4	84.1	83.4	84.4	86.5	88.2
5	90.0	90.8	88.5	89.1	87.7	88.2	89.7	94.7
6	88.5	87.7	90.8	92.0	93.2	92.6	86.2	73.5
7	72.2	74.9	59.5	57.2	53.3	44.8	27.3	26.7
8	71.9	61.2	52.6	54.2	51.2	47.0	27.7	25.0

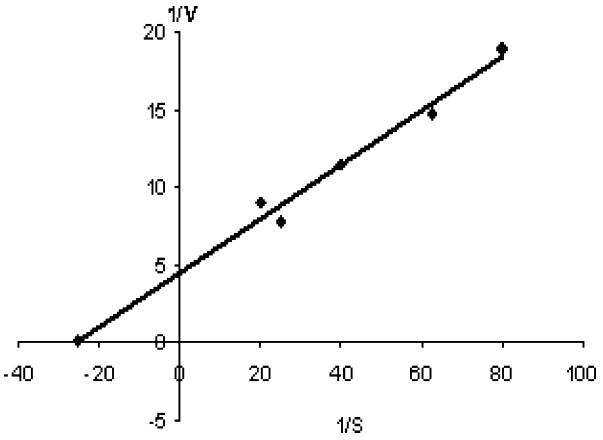


Fig. 4 Lineweaver – Burk plot of decolorization of NBB by purified HRP

Effect of Temperature

A series of experiments was carried out under the same operating conditions but varying reaction temperatures and the results are shown in Fig. 2 for comparison. As shown by Fig. 2a, at 30 °C maximum decolorization rate of NBB was obtained. As the temperature is increased from 30 °C to 70 °C, at pH 7, a slow decrease in the rate was observed. The reason may be attributed to the fact that the activity of purified HRP enzyme decreases at temperatures above 30 °C, as determined in our previous study [31]. As clearly shown in Fig. 2b, the maximum amount of degradation (80-90%) in the first 5 minutes of treatment did not change with temperature at pH 5.

Influence of pH

It was found that enzymatic decolorization of NBB was maximal at pH 4 and 5. As clearly shown in Fig. 3, decolorization reached about 80-90% at both the 5th min. of treatment for

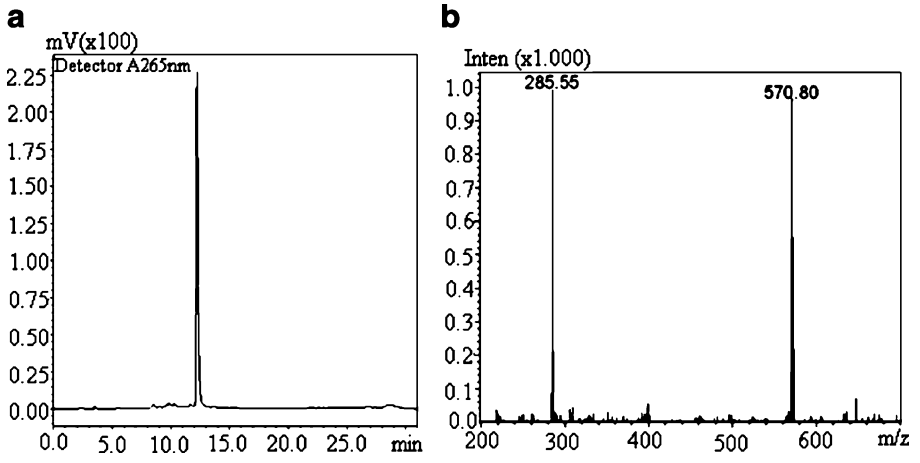


Fig. 5 **a**) HPLC chromatogram of naphtol blue black acquired from UV detector at 265 nm. **b**) Mass spectrum of naphtol blue black eluted at 12.2 min

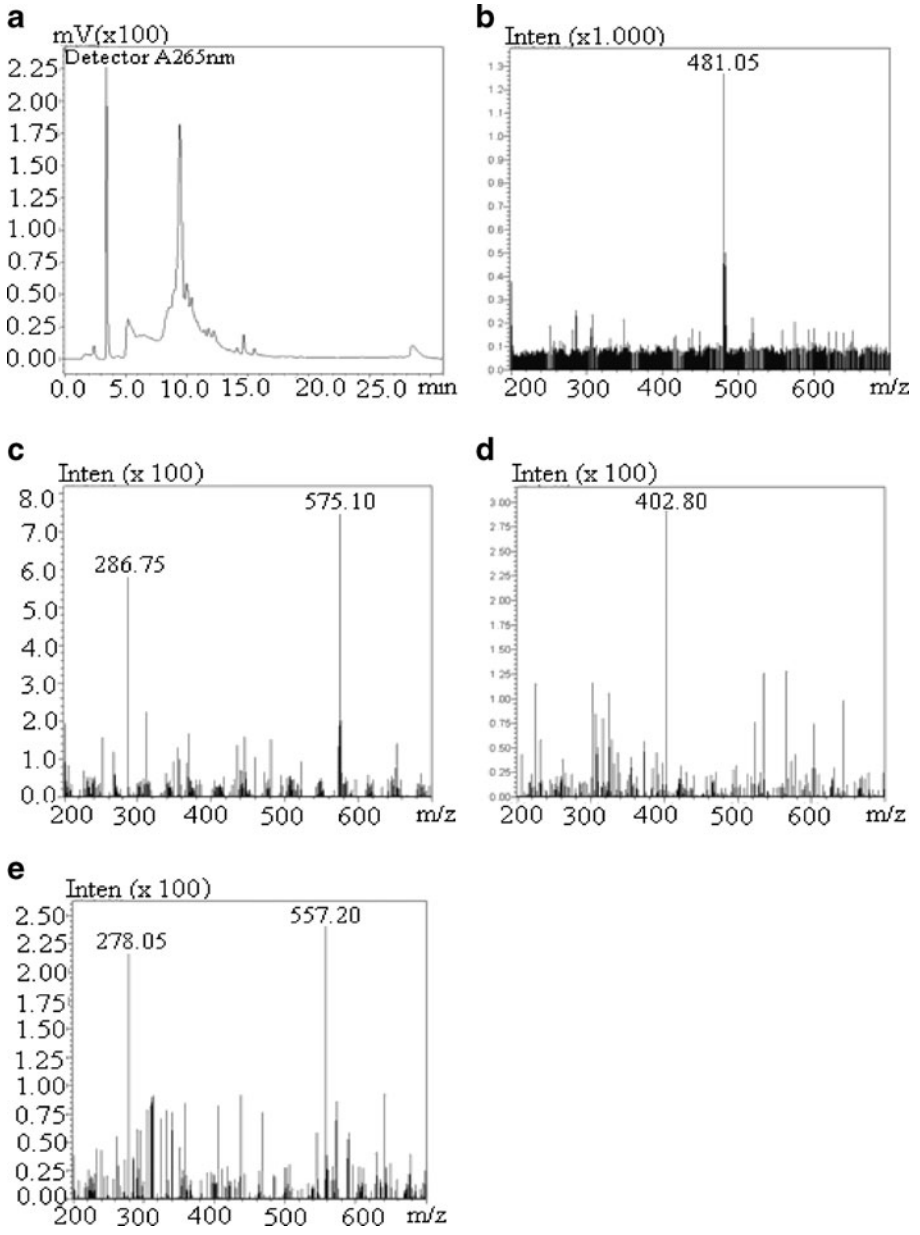


Fig. 6 HPLC chromatogram of Naphtol blue black after degradation with HRP at pH 5.0 (**a**). Mass spectra of the peaks eluted at 9.40 min. (**b**), 9.20 min. (**c**), 10.80 min. (**d**) and 10.15 min. (**e**)

pH 3-5 and the 10th min. for pH 6. This was an extremely short period to achieve azo dye degradation. In other pH values (pH 7 and 8), decolorization reached 60% after 60 minutes. The pH effect on enzymatic decolorization was associated with the pH dependence of the enzyme activity. It has been reported that HRP showed the best activity at pH 4 and 5 [33]. The results of this study are in agreement with the ones obtained by Dong et al., suggesting that decolorization might be due to the HRP activity.

Variation of dye removal at different temperature and pH values after 60 minutes of treatment are presented in Table 1. The decolorization at pH 4-6 achieved 80-90% at all temperatures. As for pH 3, 7 and 8, the decolorization reached 70-80% at 30 °C, afterwards it showed a decline with increased temperatures.

Enzyme Kinetic Data

To investigate the mechanism of enzymatic conversion, a kinetic model has been used to fit the experimental data. The correlation between specific decolorization rate and dye concentration can be described by Michaelis-Menten kinetics. A Lineweaver – Burk plot was made of the initial rates obtained at varying dye concentrations while the amount of enzyme was held constant (Fig. 4). The Michaelis-Menten constant (K_m) and maximum decolorization rate (V_m) of purified HRP were determined for NBB. The K_m and V_m values were estimated to be 39±0.2 μM and 0,227±0.01 mmol min^{-1} respectively. Error values are the calculated standart deviations (±SD, n=3).

LC-MS Analysis

HPLC chromatogram of NBB obtained from UV detector (at 265 nm) and mass spectrum of the molecule are shown in Fig. 5. Molecular weight of NBB without sodium ions is 570.51 Da. In mass spectrum, peaks of 285.55 and 570.80 belong to the molecular ions of NBB as $[M\text{-}2Na^+]^{2-}$ and $[M\text{-}2Na^+ + H^+]^-$.

HPLC chromatogram and mass spectra of degradation products of NBB are shown in Fig. 6. After 30 min. degradation of NBB with HRP at pH 5.0, a number of peaks appeared in the chromatogram (Fig. 6a). The most abundant ions after the degradation are 481.05, 286.75, 575.10, 402.80, 278.05 and 557.20. The major peak of the chromatogram has a retention time of 9.40 min. and mass spectrum of this peak is shown in Fig. 6b. Ion of 481.05 represents that the enzyme degraded the dye at the azo linkage of phenylazo linkage. Relative abundance of this molecule in degradation products is 37% according to the peak areas of HPLC chromatogram. In Fig. 6c, ions of 286.75 and 575.10, eluted at 9.20 min. indicate a molecule having a higher molecular mass, 574 Da, than the parent molecule. Hydrogenation of both azo linkages from R–N = N–R^1 to R–NH–NH–R^1 may increase the molecular mass of parent molecule to 574 Da. The ion of 402.80 belongs to a fragment of NBB, in which a sulfonate group and phenyl group of azo linkage are absent (Fig. 6d). 278.05 and 557.20 belong to the molecular ions of naphtol blue black without the amine group (Fig. 6e). These results suggest that the decolorization of dye proceed via the reductive cleavage of azo bond as shown in Fig. 7. Troupis et al. reported similar results for the Photocatalytic reductive destruction of NBB [34].

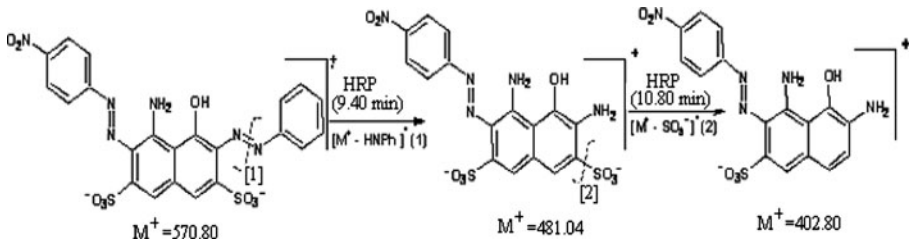


Fig. 7 Dye degradation products identified using LC–MS

Conclusion

Textile wastewaters are characterized as having a strong color, since some of the initial dye (10–15%) is not fixed to the fiber during the dyeing process, thus being released to the effluents. When released to the environment without treatment, they can cause serious contamination problems, decreasing the water transparency and, consequently, inhibiting the penetration of solar radiation and decreasing photosynthesis. The enzyme; horseradish peroxidase, showed a good decolorization of a textile diazo dye, NBB.

This paper reported some characteristics of in vitro colour degradation of one common industrial azo dye, naphtol blue black. The performance of HRP catalyzed reaction for degradation of dye was found to be dependent upon the reaction time, pH and temperature. The most suitable pH for decolorization was 4 to 5. The decolorization was very fast and was complete within a few minutes after the mixing of reaction components. At this pH values, decolorization was observed rapidly as 80-90% at all temperatures after 5 minutes (i.e., was not changed significantly with increased temperatures).

After 60 minutes of treatment, the decolorization activity was not affected by temperature changes (25-70 °C) in the range of 4-6 whereas at pH 7 and 8, the decolorization showed a decline with increased temperatures. To our knowledge this is the first study to demonstrate the ability of the HRP enzyme to decolorize NBB dye. The results show that enzymatic decolorization of NBB is quick and provides new perspectives for the use of these or related system in environmental biotechnology.

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References

1. Rocher, V., Siaugue, J. M., Cabuil, V., & Bee, A. (2008). Removal of organic dyes by magnetic alginate beads. *Water Research, 42*, 1290–1298.
2. Chen, J., & Zhu, L. (2007). Heterogeneous UV-Fenton catalytic degradation of dyestuff in water with hydroxyl-Fe pillared bentonite. *Catalysis Today, 126*, 463–470.
3. Forgacs, E., Cserhati, T., & Oros, G. (2004). Removal of synthetic dyes from wastewaters: A review. *Environment International, 30*, 953–971.
4. Robinson, T., McMullan, G., Marchant, R., & Nigam, P. (2001). Remediation of dyes in textile effluent: A critical review on current treatment Technologies with a proposed alternative. *Bioresource Technology, 77*, 247–255.
5. Patel, R., & Suresh, S. (2006). Decolourization of azo dyes using magnesium–palladium system. *Journal of Hazardous Materials, 136*, 1729–1741.
6. Sye, W. F., Lu, L. C., Tai, J. V., & Wang, J. I. (2008). Applications of chitosan beads and porous crab shell powder combined with solid-phase microextraction for detection and the removal of colour from textile wastewater. *Carbohydrate Polymers, 72*, 550–556.
7. Cioni, F., Bartolucci, G., Pieraccini, G., Meloni, S., & Moneti, G. (1999). Development of a solid phase microextraction method for detection of the use of banned azo dyes in coloured textiles and leather. *Rapid Communication in Mass Spectrometry, 13*, 1833–1837.
8. Cao, J., Wei, L., Huang, Q., Wang, L., & Han, S. (1999). Reducing degradation of azo dye by zero-valent iron in aqueous solution. *Chemosphere, 38*, 565–571.
9. Harazona, K., Watanabe, Y., & Nakamura, K. (2003). Decolorization of azo dye by the white-rot basidiomycete phanerochaete sordida and by ıts manganese peroxidase. *Journal of Bioscience and Bioengineering, 95*, 455–459.
10. Novotny, C., Rawal, B., Bhatt, M., Patel, M., Susek, V., & Molutoris, H. P. (2001). Irpex lactless and Pleatotus ostreatus for decolourization of chemically different dyes. *Journal of Biotechnology, 89*, 113–122.

11. Glenn, J. K., & Gold, M. H. (1983). Decolorization of several polymeric dyes by the lignin degrading basidiomycete Phanerochaete chrysosporium. *Applied and Environmental Microbiology, 45*, 1741–1747.
12. Manimekalai, R., & Swaminathan, T. (2000). Removal of hazardous compounds by lignin peroxidase from Phanerochaete crysospori. *Bioprocess Engineering, 22*, 29–33.
13. Nicel, J. A. (1994). Kinetics of horseradish peroxidase catalyzed polymerization and precipitation of aqueous 4-chlorophenol. *Journal of Chemical Technology and Biotechnology, 60*, 203–215.
14. Koller, G., Moder, M., & Czihal, K. (2000). Peroxidative degradation of selected PCB co mechanistic study. *Chemosphere, 41*, 1827–1834.
15. Bhunia, A., Susheel, D., & Pramod, P. W. (2001). Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnology and Bioengineering, 72*, 562–567.
16. Mohan, S. V., Prasad, K. K., Rao, N. C., & Sarma, P. N. (2005). Acid azo dye degradation by free and immobilized horseradish peroxidase (HRP) catalyzed process. *Chemosphere, 58*, 1097–1105.
17. Deveci, T., Unyayar, A., & Mazmanci, M. A. (2004). Production of remazol brilliant blue R decolourising oxygenase from the culture filtrate of Funalia trogii ATCC 200800. *Journal of Molecular Catalysis. B, Enzymatic, 30*, 25–32.
18. Okazaki, S., Nagasawa, S., Goto, M., Furusaki, S., Wariishi, H., & Tanaka, H. (2002). Decolorization of azo and anthraquinone dyes in hydrophobic organic media using microperoxidase-11 entrapped in reversed micelles. *Biochemical Engineering Journal, 12*, 237–241.
19. Aitken, M. D. (1993). Waste treatment applications of enzymes: opportunities and obstacles. *Chemical Engineering Journal, 52*, B49–B58.
20. Klibanov, A. M., Tu, T. M., & Scott, K. P. (1983). Peroxidase-catalyzed removal of phenols from coal conversion waste waters. *Science, 221*, 259–261.
21. Malony, S. W., Manem, J., Mallevialle, J., & Fiessinger, F. (1986). Transformation of trace organic compounds in drinking water by enzymatic oxidative coupling. *Environmental Science & Technology, 20*, 249–252.
22. Buchanan, I. D., & Nicel, J. A. (1999). A simplified model peroxidase catalysed phenol removal from aqueous solution. *Journal of Chemical Technology and Biotechnology, 74*, 669–674.
23. Wagner, M., & Nicel, J. A. (2002). Impact of dissolved wastewater constituents on peroxidase catalyzed treatment of phenol. *Journal of Chemical Technology and Biotechnology, 77*, 419–428.
24. Arseguel, D., & Baboulene, M. (1994). Removal of phenol from coupling of talc and peroxidase. Application of depollution of wastewater containing phenolic compounds. *Journal of Chemical Technology and Biotechnology, 61*, 331–335.
25. Putter, J., & Becker, R. (1983). *Peroxidases, methods of enzymatic analysis* (pp. 286–293). Weinheim: Verlag Chemie.
26. Sudoh, M., Kodera, T., Sakai, K., Zhang, J. Q., & Koide, K. (1986). Oxidative degradation of aqueous phenol effluent with electrogenerated fenton's reagent. *Journal of Chemical Engineering Japan, 19*(6), 513–518.
27. Min Zhu, M., Huang, X., & Shen, H. (2001). Aromatic azo compounds as spectrophotometric kinetic assay substrate for HRP. *Talanta, 53*, 927–935.
28. Liu, J. Z., Song, H. Y., Weng, L. P., & Ji, L. N. (2002). Increased thermostability and phenol removal efficiency by chemical modified horseradish peroxidase. *Journal of Molecular Catalysis. B, Enzymatic, 18*, 225–232.
29. Kim, G. Y., Lee, K. B., Cho, S. H., Shim, J., & Moon, S. H. (2005). Electroenzymatic degradation of azo dye using an immobilized peroxidase enzyme. *Journal of Hazardous Materials, 126*, 183–188.
30. Luo, J., & Hepel, M. (2001). Photoelectrochemical degradation of naphthol blue black diazo dye on WO3 film electrode. *Electrochimica Acta, 46*, 2913–2922.
31. Altikatoglu, M., Arioz, C., Basaran, Y., & Kuzu, H. (2009). Stabılızatıon of horseradısh peroxıdase by covalent conjugatıon wıth dextran aldehyde agaınst temperature and pH changes. *Central European Journal of Chemisrty, 7*, 423–428.
32. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–54.
33. Dong, Y., Chen, J., Li, C., & Zhu, H. (2007). Decoloration of three azo dyes in water by photocatalysis of Fe (III)-oxalate complexes/H2O2 in the presence of inorganic salts. *Dyes and Pigments, 73*, 261–268.
34. Troupis, A., Gkika, E., Triantis, T., Hiskia, A., & Papaconstantinou, E. (2007). Photocatalytic reductive destruction of azo dyes by polyoxometallates: Naphthol blue black. *Journal of Photochemistry and Photobiology A: Chemistry, 188*, 272–278.