Redox Mediated Decolorization and Detoxification of Direct Blue 80 by Partially Purified Ginger (*Zingiber officinale*) Peroxidase

Misha Ali[1], Masood Ahmad[2], Qayyum Husain[3*]

Department of Biochemistry, Faculty of Life Science, Aligarh Muslim University, Aligarh-202002 (UP), India

***Abstract*—** Textile industries are releasing a large number of toxic synthetic dyes into waste waters. Hence, the removal of such compounds from environment prior to their final disposal is necessary. In the present study, potential use of ginger (*Zingiber officinale*) peroxidase in decolorization and detoxification of direct blue 80 has been investigated. It was found that only 0.166 U/ml of ginger peroxidase was sufficient for maximum decolorization of dye (25 mg/L). H_2O_2 was required in low concentration (0.3 mM) in the presence of 0.6 mM 1-hydroxybenzotriazole. Direct blue 80 was also successfully removed in stirred batch process. It was observed that ginger peroxidase was highly stable over a wide range of pH and temperatures. K_m and V_{max} of the enzyme for direct blue 80 was found to be 27.8 mg/L and 2.09 mg/L/min, respectively. In UV-visible spectral analysis a sharp decline in peak was observed for the treated direct blue 80 which substantiates the breakdown of chromophore group of dye. Genotoxicity assessment by comet assay and chromosomal aberration test confirmed that the direct blue 80 was successfully detoxified by ginger peroxidase. Other direct and acid dyes were also treated either as a single or a mixture of different dyes and it was observed that these dyes were also decolorized significantly under similar experimental conditions. Our study suggests that this enzyme-redox mediator system constitutes a cost effective model which can decolorize the industrial textile effluents and also can reduce the toxic load of environment.

Keywords*— *Decolorization; peroxidase, 1-hydroxybenzotriazole, Zingiber officinale

Abbreviations: DB 80, direct blue 80; DY 4, direct yellow 4; DY 50, direct yellow 50; DR 23, direct red 23; AB 1, acid black 1; AB 210, acid black 210; AY 42, acid yellow 42; GP, ginger peroxidase; HOBT, 1-hydroxybenzotriazole; PBS, phosphate buffer saline; MI, mitotic index; MMS, methyl methane sulphonate; CA, chromosomal aberrations.

I. INTRODUCTION

Textile industries are most concerned problem of the environment, because they release a wide range of colored pollutants into wastewater. Around 5-40% of the total dyes are released in the environment. [1] Colored water obstructs the absorption of sunlight and thus it can hamper the photosynthetic activity of the aquatic plants.[2] Some of the dyes are the derivatives of the benzidine, which is a potential human carcinogen.[3] Therefore, the removal of such harmful aromatic compounds is an urgent need of time. Many conventional methods have been employed for the treatment of such pollutants which involves physico-chemical and chemical processes like adsorption, chemical reduction, coagulation, electrolysis and precipitation.[4] But these methods have several inherent problems such as applicability to limited concentration ranges, high cost, incomplete removal and introduction of hazardous materials to the environment.[5] In conventional biological method, microbes have been utilized for the treatment of such compounds in wastewater.[6] However, microbial method have also many demerits like acclimatization of microbes in the dye effluent, need of substrate to support the microbial culture and production of large amount of solid wastes.[7] Recently, enzymatic method is gaining much interest for the removal of aromatic compounds from the contaminated effluents due to their merits over the other known classical and biological methods.[8]

Enzymatic methods have a number of advantages over the known conventional treatments like operation at high and low contaminant concentration; applicable over the wide range of pH, temperature and salinity; short time of treatment; reduction in sludge volume and minimum compounds are required.[9] Enzymes involved in the removal of aromatic compounds from various contaminated sites are mainly oxidoreductive enzymes such as peroxidases and polyphenol oxidases.[10] Several dyes are recalcitrant to the action of peroxidase, therefore redox mediators are required alongwith enzyme for the removal of colored pollutants.[11] Peroxidases used for the removal of dyes have been isolated from many plant sources like horseradish roots, whiteradish, turnip roots, tomato, soybean, bitter gourd and *Saccharum uvarum*.[7] However, due to high cost of purification and low enzyme activity, these enzymes have not been utilized at a large scale.[12] To ensure the removal of toxic pollutants from wastewater, ecotoxicological tests via biological assays are required.[13] Most of the animal assays are costly, therefore they are not used in routine monitoring.[14] Plant bioassays are less expensive and have been used to monitor the environmental pollution.[15]

In the present study an attempt has been made to utilise the partially purified ginger peroxidase (GP) for the removal of DB 80 which is a benzidine based dye and expected to be metabolized into benzidine which is a known human carcinogen.[3] The decoloriztion of DB 80 has been done under different optimized parameters like pH; temperature; time; concentration of enzyme, H_2O_2 and HOBT. In order to evaluate the affinity of enzyme for DB 80, kinetic parameters of the GP were determined. UV-visible spectral analysis was also done to confirm the breakdown of chromophoric group. Genotoxic assessment test was performed for DB 80 and its decolorized product by comet assay and *Allium cepa* chromosomal aberration assay. Some other dyes and their mixtures were also treated under identical treatment conditions.

II. MATERIAL AND METHODS

2.1 Chemicals

o-dianisidine-HCl, Histopaque 1077 and RPMI 1640 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulphate and 1-hydroxybenzotriazole (HOBT) were obtained from SRL Chemicals (Mumbai, India). Direct Red 23 (DR 23), Direct Blue 80 (DB 80), Direct Yellow 50 (DY 50), Direct Yellow 4 (DY 4), Acid Black 1 (AB 1), Acid Black 210 (AB 210), and Acid Yellow 42 (AY 42) were the products of Atul Chem. Co. (Valsad, Gujarat, India). Ginger was collected from local vegetable markets surroundings of Aligarh Muslim University, Aligarh, India. Most of other used reagents and chemicals were of analytical grade.

2.2 Ammonium sulphate fractionation of ginger proteins

Ginger (250 g) was homogenised in 500 mL of 0.1 M sodium acetate buffer, pH 5.5 and homogenate was filtered through four layers of cheese cloth. Filtrate was then centrifuged at 12,000 × *g* on a Remi C-24 Cooling Centrifuge for 20 min at 4°C. The obtained clear solution was subjected to salt fractionation by adding 10-90% (w/v) $(NH_4)_2SO_4$. The solution was stirred overnight at 4 ºC for complete precipitation of proteins. The precipitate was collected by centrifugation at 15,000 × *g* on a Remi C-24 Cooling Centrifuge. The collected precipitate was dissolved in 0.1 M sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer.

2.3 Preparation and treatment of synthetic dye solutions

Seven different synthetic textile dyes (DR 23, DB 80, DY 50, DY 4, AB 1, AB 210, and AY 42) were solubilized in distilled water. Each dye solution (25 mg/L, 5.0 mL) were incubated independently with GP (0.166 U/mL) in sodium acetate buffer, pH 5.0, in the presence of 0.6 mM of HOBT and 0.3 mM of H_2O_2 at 40 °C for 1 h. The reaction was stopped by keeping in a boiling water bath for 5 min. The decolorization of dye was monitored spectrophotometrically by measuring decrease in absorbance at the specific wavelength maximum, λ_{max} for each dye on UV-1700 PharmaSpec UV–vis spectrophotometer. Untreated dye solution containing all the reagents that are present in treated solutions except the enzyme was used as control (100%) for the calculation of percent decolorization. The parameter percent dye decolorization was calculated as:

$$r = (A_u - A_t / A_u) \times 100$$

where r is the percent decolorization (%), A_u is absorbance of the untreated dye, and A_t is absorbance of treated dye.

2.4 Optimization of different parameters for maximum decolorization of DB 80

2.4.1 Effect of GP concentration

Five millilitre solution of DB 80 was treated with increasing concentrations of GP (0.0208~0.2080 U/mL) in 0.1 M sodium acetate buffer, pH 5.0 in the presence of 0.3 mM H_2O_2 and 0.6 mM HOBT for 1 h at 40°C. The reaction was terminated by putting in a boiling water bath and intensity of color was measured.

2.4.2 Effect of HOBT

Five milliliter solution of DB 80 was treated with increasing concentrations of HOBT (0.10~1.0 mM) in 0.1 M sodium acetate buffer, pH 5.0 in presence of GP (0.166 U/mL) and 0.3 mM H_2O_2 for 1 h at 40°C. The reaction was terminated by heating and dye decolorization was monitored.

2.4.3 Effect of H_2O_2

Five millilitre solution of DB 80 was treated with increasing concentrations of H_2O_2 (0.15~1.5 mM) in 0.1 M sodium acetate buffer, pH 5.0 in presence of GP (0.166 U/mL) and 0.6 mM HOBT for 1 h at 40°C. The reaction was terminated by heating and dye decolorization was monitored.

2.4.4 Effect of pH and temperature

DB 80 solutions were prepared in the buffers (0.1 M) of different pH (2.0~9.0). These buffers were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 ~7.0), and Tris-HCl (pH 8.0 and 9.0). Each dye solution (5.0 mL) was treated with GP (0.166 U/mL) in buffers of varying pH (3.0~9.0) in the presence of 0.3 mM H_2O_2 and 0.6 mM HOBT for 1 h at 40°C. The reaction was terminated by heating in a boiling water bath for 5 min. The decolorization of dye was monitored at its λ_{max}.

Five millilitre solution of DB 80 was treated with GP (0.166 U/mL) at different temperatures (20~80°C) under other conditions specified above. Reaction was terminated by incubating in a boiling water-bath for 5 min. Absorbance of remaining color was measured at its λ_{max}.

2.4.5 Effect of time on GP mediated decolorization of DB80

Five millilitre solution of DB 80 was treated with GP (0.166 U/mL) in the presence of 0.3 mM H_2O_2 and 0.6 mM HOBT in 0.1 M sodium acetate buffer, pH 5.0 at 40°C for varying time intervals. The reaction was terminated by heating for 5 min. The dye decolorization was monitored at 580 nm.

2.5 Determination of K_m and V_{max} of GP

The initial rates of decolorozation of DB 80 were determined at different concentrations ranging from 5 mg/L-100 mg/L, in 0.1 M sodium acetate buffer, pH 5.0 in the presence of 0.3 mM H_2O_2 and 0.6 mM HOBT at 40°C. K_m and V_{max} were determined from linearization of Michaelis-Menten equation.

2.6 UV–visible spectral analysis

The absorption spectra of treated and un-treated DB80 solutions were recorded on UV-1700 PharmaSpec UV–vis spectrophotometer.

2.7 Treatment of synthetic dye solutions and their mixtures

Different mixtures of dyes were prepared by mixing independent dyes in equal proportion to the final concentration of 25 mg/L. The mixtures of dyes were treated with GP (0.166 U/mL) in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.3 mM H_2O_2 and 0.6 mM of HOBT for 1 h at 40°C. Decrease in absorbance in each GP treated polluted water was monitored at each specific wavelength maxima of the mixture.

2.8 Treatment of DB 80 in stirred batch process

Decolorization of DB 80 in stirred batch process was observed spectrophotometrically. DB 80 (50 mg/L, 250 mL) was treated with 12.5 U of GP in the presence of 0.3 mM of H_2O_2 and 0.6 mM of HOBT in continuous stirring conditions for 2 h. Aliquots were taken after each time interval of 15 min and absorbance was monitored at maximum wavelength.

2.9 Genotoxicity assessment of DB 80 and its decolorized product by comet assay

2.9.1 Isolation of lymphocytes

Heparinized blood sample (10 mL) from single healthy donor was obtained by venupuncture and diluted suitably in $Ca^{2+}$and Mg^{2+} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma, USA) and the cells were finally suspended in RPMI 1640. The donor (first author) donated blood for all experiments.

2.9.2 Experimental procedure

Comet assay was performed under alkaline conditions according to the procedure of Singh et al.[16] with slight modifications. Fully frosted microscopic slides pre-coated with 1% normal melting agarose. Diluted lymphocytes were then mixed properly with equal volume of 2.0% low melting agarose and half of the mixture was pipetted over the first layer. The slides were covered immediately by cover slips and placed on ice for 15 min to solidify. Immediately after removing the cover slips, within 20 min cells were treated with increasing concentrations of DB 80 and its decolorized product (25 mg/L, 50 mg/L and 100 mg/L) for 1 h at 4ºC. Lysis was done in cold solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10 and 1% Triton X-100 (added just prior to use) for 1 h at 4ºC followed by unwinding of DNA for 30 min in alkaline electrophoretic solution containing 300 mM NaOH, 1 mM EDTA, pH 13 (at 4ºC, 300 mA current). After electrophoresis,

DNA was neutralized by 0.4 M Tris, pH 7.5. The slides were stained with 75 µl ethidium bromide (EtBr, 20 µg/mL), washed with distilled water, covered with cover slips and placed in a humidified chamber.

2.9.3 Visualization of slides and scoring

Slides were visualized using an image analysis system (Komet 5.5; Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and a COHU 4910-integrated CC camera (equipped with 510–560 nm excitation and 590 nm barrier filters) (COHU, San Diego, USA). Images from 50 cells (25 from each replicate slide) were analyzed. Tail length (migration of DNA from the nucleus in µ meter) was chosen as the parameter to assess DNA damage.

2.10 Allium cepa chromosomal aberration assay

Allium cepa chromosomal aberration test was performed according to Fiskesjo (1985).[17] Small bulbs (1.5-2.0 cm in diameter) of the common onion, *Allium cepa*, (2n=16) were taken and the outer scales of the bulbs were removed without destroying the root primordia. Bulbs of *Allium cepa* were placed in test tubes with their basal ends dipping in distilled water and germinated at room temperature (25 ± 2˚C). When the newly emerged roots were of 1.00-2.00 cm in length, they were used in the test. Roots of *Allium cepa* were treated with 50 mg untreated and treated DB 80. Distilled water and methyl methane sulphonate (MMS) (10 mg/l) were used as negative and positive controls, respectively. After 72 h of exposure, several root tips were removed from the bulbs, fixed in 3:1 ethanol: glacial acetic acid and stored overnight at 4°C. The next day, they were rinsed in tap water and stained in acetocarmine. The squash technique was applied for the study of the mitotic index (MI) and chromosomal aberrations (CA). For each water sample, microscopy was performed on three replicate slides which contained 3-4 root tips. To obtain MI, approximately 3000 cells (1000 cells in each of the three slides) were observed for each water sample. The number of CA was recorded in approximately 300 dividing cells (preferably 100 per slide). Several types of CA were analyzed within different cell division stages (prophase, metaphase, anaphase and telophase).

2.11 Measurement of peroxidase activity

Peroxidase activity was measured from the change in the optical density (λ_{460} nm) at 37°C by measuring initial rate of oxidation of *o*-dianisidine-HCl in presence of H_2O_2, using both substrates in saturating concentrations.[9]

One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1 µmol of *o*-dianisidine-HCl in presence of H_2O_2 per min at 37°C into coloured product (ε_m= 1.13×10^4 M^{-1} cm^{-1}, where $\varepsilon_m$is molar extinction coefficient).

2.12 Determination of protein concentration

The protein concentration was determined by the procedure of Lowry et al. (1951).[18] Bovine serum albumin was used as the standard.

III. RESULTS

3.1 Optimization of different parameters for maximum decolorization of DB 80

Fig. 1a demonstrates the effect of increasing concentration of GP on decolorization of DB 80. There was continuous enhancement in decolorization of DB 80 with the addition of increasing concentration of GP. However, maximum decolorization was obtained at 0.064 U/mL concentration of GP. No further increase was observed above 0.166 U/mL of enzyme.

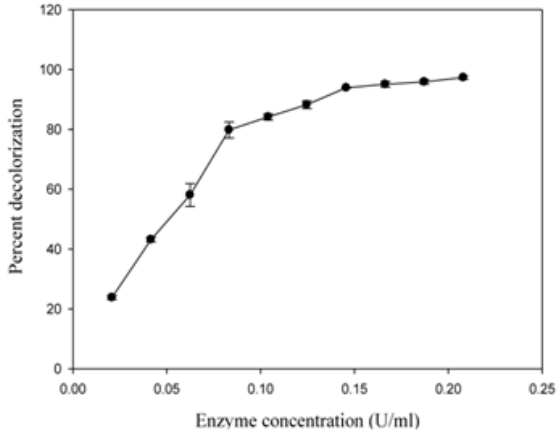
Effect of increasing concentration of HOBT on the decolorization of DB 80 has been evaluated (Fig. 1b). Decolorization of DB 80 was continuously enhanced up to 0.6 mM HOBT and above this concentration no remarkable change was noticed. Therefore, maximum decolorization (97%) of DB 80 was obtained at 0.6 mM HOBT.

Maximum decolorization (97%) of DB 80 was obtained only at 0.3 mM H_2O_2 which remained constant upto 0.6 mM H_2O_2 and then slight decrease in decolorization was observed from 0.75 mM - 1.5 mM H_2O_2 (Fig. 1c).

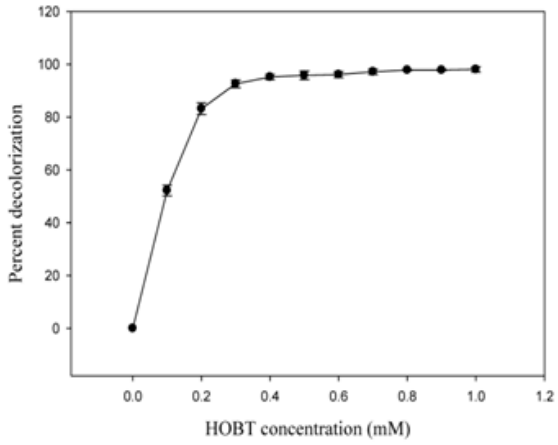
DB 80 was treated with GP (0.166 U/mL) in buffers of different pH (2.0 ~ 10.0). Results showed that GP can decolorize the dyes only in acidic pH with slight decolorization at pH 3.0 (29%) and very high at pH 4.0 (94%), pH 5.0 (96%) and pH 6.0 (95%). An abrupt decrease in decolorization was observed at pH 7.0 (Fig. 1d).

Treatment of DB 80 at different temperatures was analysed in presence of GP (0.166 U/mL). More than 90% decolorization was observed over a wide range of temperatures (20 °C-50 °C). A significant amount of DB 80 was also decolorized at 60°C, while a sudden decrease in decolorization was observed at 70 °C (Fig. 1e).

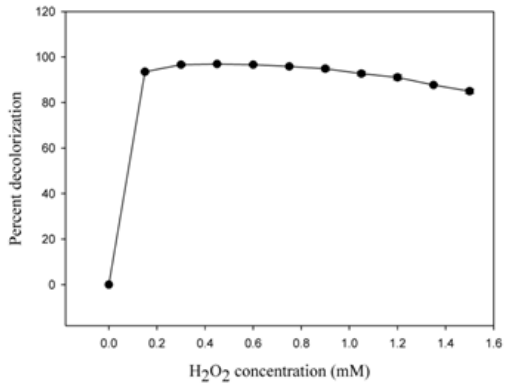
Fig. 1f demonstrates that maximum decolorization of DB 80 was obtained within 1 h at 40°C. No further decolorization of DB 80 was recorded when incubated for longer periods. A sufficient amount of DB 80 was decolorized only within 15 min.



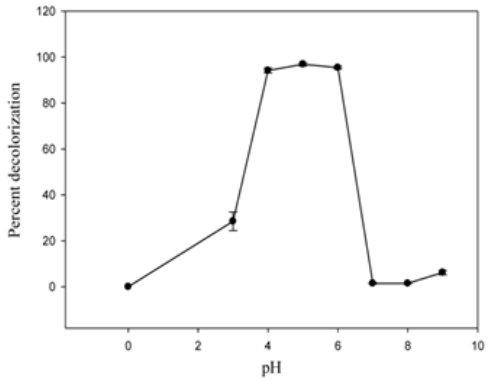
(a) EFFECT OF ENZYME CONCENTRATION ON DECOLORIZATION OF DB 80



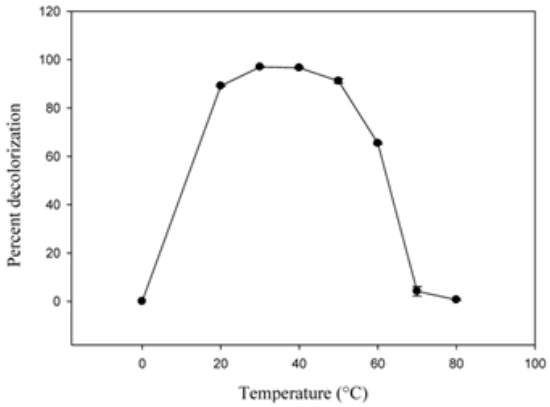
(b) EFFECT OF HOBT ON DECOLORIZATION OF DB 80 BY GP



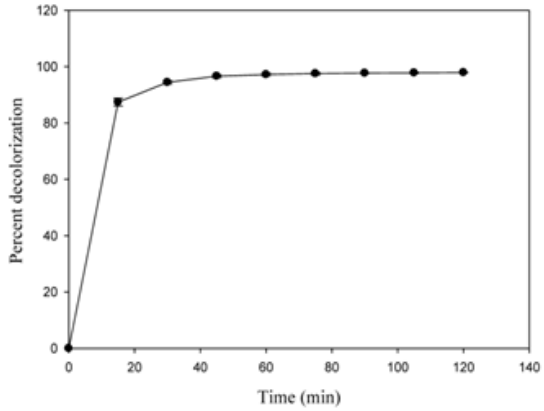
(c) EFFECT OF H_2O_2 ON DECOLORIZATION OF DB 80 BY GP



(d) EFFECT OF pH ON THE DECOLORIZATION OF DB 80



(e) EFFECT OF TEMPERATURE ON THE DECOLORIZATION OF DB 80



(f) EFFECT OF TIME ON DECOLORIZATION OF DB 80.

FIGURE 1

3.2 Determination of K_m and V_{max} of GP for DB 80

In order to determine the K_m and V_{max} of GP for DB 80 an experiment was performed with dye concentrations ranging from 5mg/L to 100 mg/L. The plot of initial rate versus dye concentration followed the hyperbolic pattern according to Michaelis-Menten kinetics (Fig. 2). The V_{max} and K_m of GP for DB 80 were found to be 2.09 mg/L/min and 27.8 mg/L, respectively.

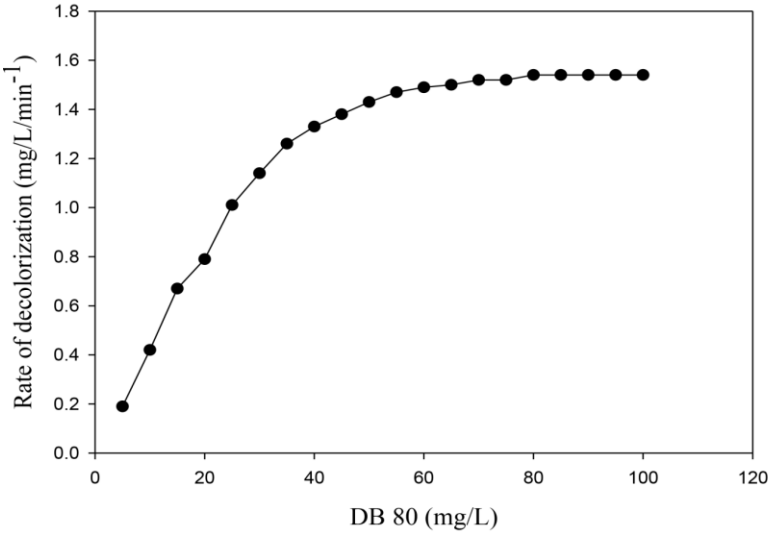


FIG. 2: MICHAELIS-MENTEN CURVE.

3.3 UV–visible spectral analysis

UV-visible spectra for treated and untreated DB 80 were monitored on UV-1700 Pharma Spec UV-visible spectrophotometer. Diminuation of peak in visible region was observed for treated DB 80 due to removal of chromophoric group (Fig. 3).

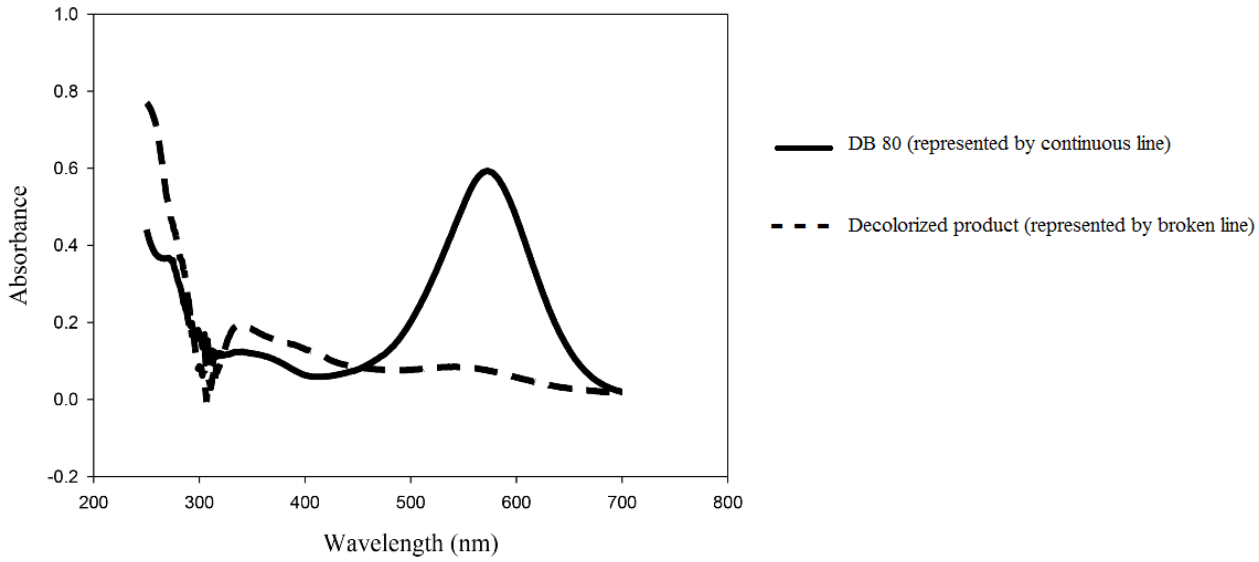


FIG. 3:UV-VIS ABSORPTION SPECTRA OF DB 80.

3.4 Treatment of synthetic dye solutions and their mixtures

Table 1 shows that most of the dyes were recalcitrant to the action of GP but in the presence of HOBT, a redox mediator a significant amount of all the synthetic dyes were decolorized. AB 210 and DY 50 were decolorized less than 50% while DB 80 was decolorized to a greater extent and the decolorization of other synthetic dyes was in between 70% to 90%. Complex mixtures of dyes were also decolorized quite fast under the optimized conditions. About 49~80% of complex mixtures of dyes were decolorized by GP (Table 2).

TABLE 1
TREATMENT OF VARIOUS DYES BY GP

Name of dyes	λ_{max} (nm)	Decolorization (%)	
		HOBT (-)	HOBT (+)
DB 80	580	8.5 ± 1.10	95.5 ± 0.45
DR 23	507	0	89.8 ± 0.45
DY 4	397	37 ± 0.55	72.2 ± 0.20
DY 50	395	0	46.2 ± 0.30
AB 1	620	0	84.6 ± 0.20
AB 210	610	0	38.2 ± 0.75
AY 42	409	0	88.2 ± 0.85

TABLE 2
TREATMENT OF DIFFERENT MIXTURES OF DYES BY GP

Mixtures	Composition	λ_{max} (nm)	Decolorization (%)
A	DR 23 + DY 4 + DB 80 + AB 210	429	63.6 ± 0.55
B	DR 23 + DB 80 + AY 42 + DY 50	407	49.4 ± 1.16
C	DY 50 + AB 210 + DY 4 + AB 1	618	70.7 ± 0.45
D	AB 1 + DB 80 + DY 4 + AY 42	596	81.6 ± 0.30
E	AB 1 + DR 23 + AB 210 + AY 42	617	69.5 ± 0.26
F	DR 23 + DY 4 + DB 80 + DY 50 + AB 210 + AB 1 + AY 42	590	74.1 ± 1.85

3.5 Treatment of DB 80 in stirred batch process

It can be observed from the results (Table 3) that around 65% of DB 80 was decolorized just within 15 min in stirred batch process. Maximum decolorization (98.3%) was obtained only in 1h and after that no further decolorization was occurred.

TABLE 3
TREATMENT OF DB 80 IN STIRRED BATCH PROCESS

Time (min)	Decolorization (%)
15	65.0 ± 1.23
30	88.5 ± 2.17
45	96.0 ± 2.11
60	98.3 ± 1.85
75	98.5 ± 2.34
90	98.5 ± 1.98
105	98.6 ± 1.03
120	98.6 ± 1.73

3.6 Genotoxic assessment of DB 80 and its decolorized product

Genotoxic assessment by comet assay was done for different concentrations of DB 80 (25 mg/L, 50 mg/L and 100 mg/L) and was compared with same concentrations of decolorized product under similar experimental conditions. The results showed that DB 80 was significantly genotoxic as compared to its decolorized products (Fig. 4, 5).

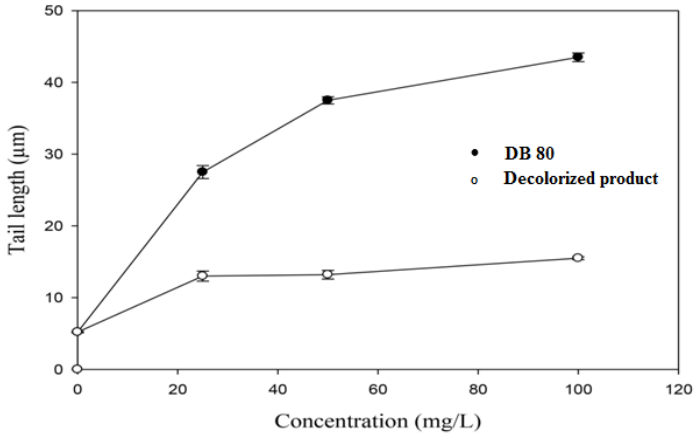


FIG. 4: COMET ASSAY FOR DB 80 AND ITS DECOLORIZED PRODUCT.

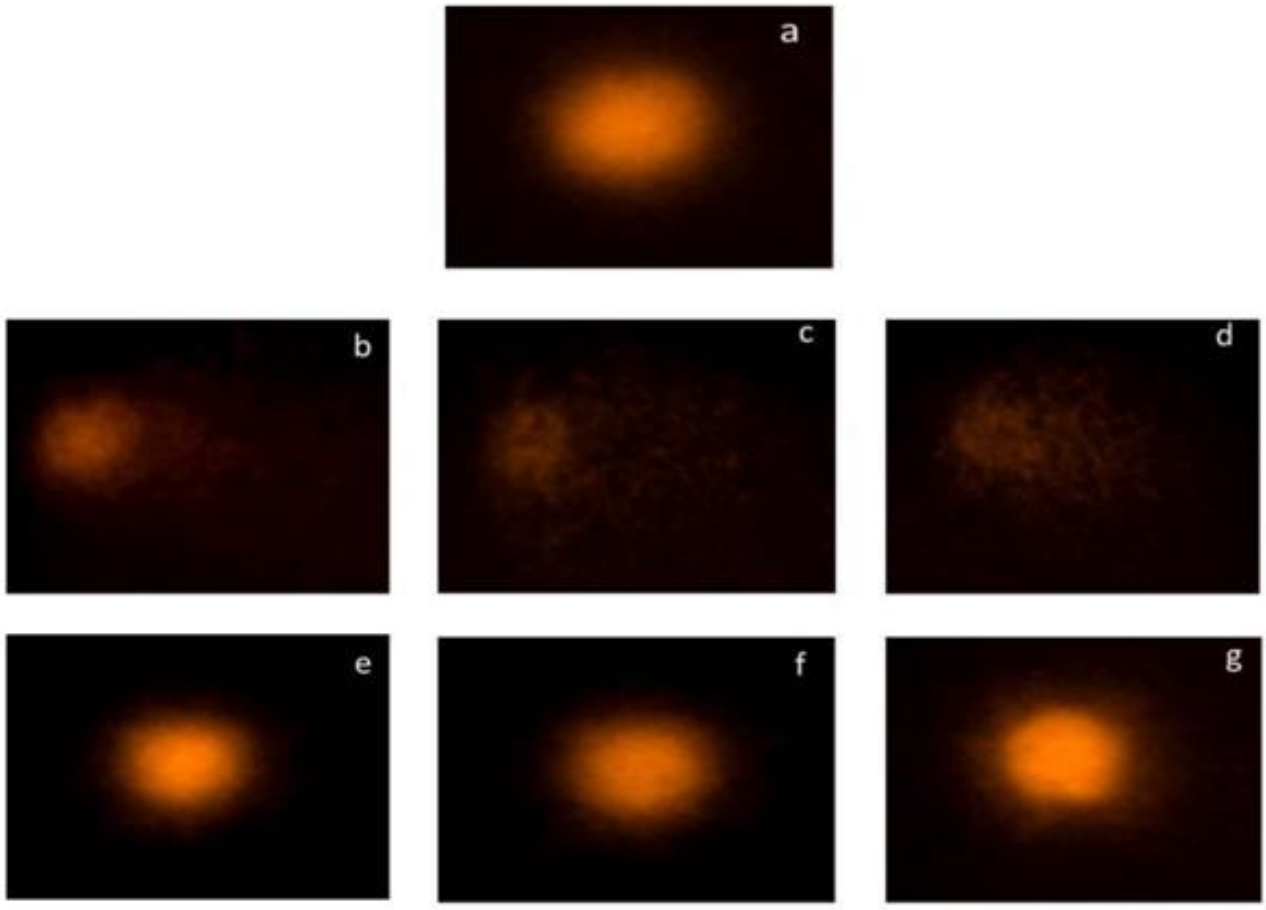


FIG. 5: IMAGES OF COMET ASSAY FOR GENOTOXIC ASSESSMENT OF DB 80 AND ITS DECOLORIZED PRODUCT.(a) CONTROL, (b-d) DB 80 [25 mg/L, 50 mg/L, 100 mg/L respectively], (e-g) DECOLORIZED PRODUCT [25 mg/L, 50 mg/L, 100 mg/L RESPECTIVELY].

3.7 Allium cepagenotoxicity test

Table 4 depicts the effects of untreated and treated DB 80 on mitotic index (MI) and the frequency of mitotic phases of *Allium cepa* root meristem cells. It can be seen that MI was significantly increased after the treatment of DB 80 by GP. Negative control (distilled water) exhibited the highest MI (31.43%), while cells exposed to methyl methane sulphonate (MMS) had the lowest average (11.47%). It was noted that the rate of each of the mitotic stages in *Allium cepa* was affected after treatment. The percentage of the prophase stage was decreased while the percentages of metaphase and anaphase-telophase stages were increased after treatment with DB 80, indicating the decrease in anti-proliferative activity.

TABLE 4
EFFECT OF UNTREATED AND TREATED DB 80 ON MITOTIC INDEX AND MITOTIC PHASE OF *ALLIUM CEPA* ROOT MERISTEM CELLS

Sample	Mitotic phases (%)			Mitotic index (% ± SD)
	Prophase	Metaphase	Anaphase-Telophase	
Untreated DB 80	56.9	20.57	24.4	17.98 ± 1.76 b
Treated DB 80	28.01	28.7	33.57	28.25 ± 3.35 a
Positive control	69.05	15.18	15.9	11.47 ± 2.1 c
Negative control	25.3	29.8	44.81	31.43 ± 0.95 a

Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test); ±: Standard deviation

In the chromosomal aberration test conducted with root meristem cells of *Allium cepa*, the negative control exhibited few abnormalities when compared with the positive control and the treated sample (Table 5). The total mitotic abnormalities were decreased after treatment. Based on the statistical analysis using the Duncan multiple range test ($p<0.05$), significant results were found. The types of aberration caused by DB 80 are C-mitosis, and a phase bridge, stickiness, laggard, vagarant, disturbed metaphase and disturbed anaphase (Fig. 6).

TABLE 5
CHROMOSOME ABERRATION IN THE ROOT MERISTEM CELLS OF ALLIUM CEPA EXPOSED TO UNTREATED AND TREATED DB 80 FOR 72 H

Sample	Types of aberrations							Total aberrant cells %
	CM	AB	L	V	S	DM	DA	
Untreated DB 80	11	20	2	6	16	6	2	21 b
Treated DB 80	1	5	2	3	2	-	1	5 c
Positive control	21	32	5	7	31	9	9	38 a
Negative control	1	2	-	2	2	-	-	2.3 c

CM: C-mitosis, AB: anaphase bridge, L: laggard, V: vagarant, S: stickiness, DM: disturbed metaphase, DA: disturbed anaphase.
Means with the same letters do not significantly differ at 0.05 level (Duncan multiple range test)

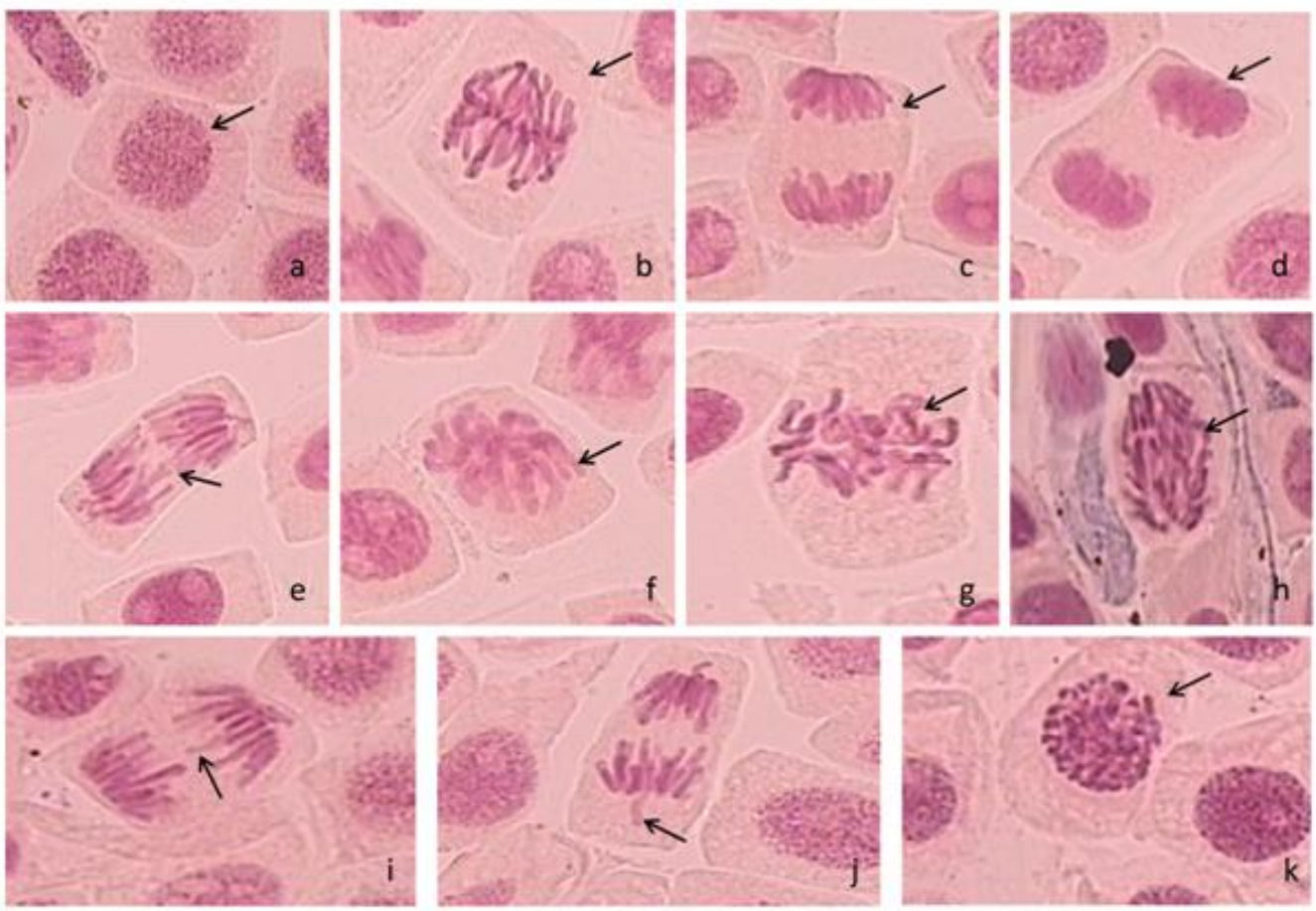


FIG. 6: DIFFERENT TYPES OF CHROMOSOMAL ABERRATIONS INDUCED BY DB 80 IN ALLIUM CEPA ROOT TIPS. (a) PROPHASE; (b) METAPHASE; (c) ANAPHASE; (d) TELOPHASE; (e) ANAPHASE BRIDGE; (f) C-MITOSIS; (g) DISTURBED METAPHASE; (h) DISTURBED ANAPHASE; (i) LAGGARD CHROMOSOME; (j) VAGARANT CHROMOSOME; AND (k) STICKINESS.

IV. DISCUSSION

Many textile industries are employing synthetic dyes to color different kind of fabrics. These synthetic dyes are comprised of complex aromatic structure and hence resistant in nature.[19] Therefore their degradation is of great challenge. Many plant peroxidases have been employed for the treatment of textile dyes.[20] In the present study an attempt has been made to partially purify the peroxidase from ginger, which is an inexpensive and easily available plant source. Peroxidase was isolated by ammonium sulphate fractionation which showed specific activity of 2.46 U/mg. Different parameters like pH, temperature, time, concentration of enzyme; H_2O_2; and HOBT were optimized for the maximum decolorization of dyes. HOBT is a redox mediator which is helping in the removal of recalcitrant aromatic pollutants from wastewater.[21]

The maximum decolorization was obtained at 0.166 U/ml of GP which is significantly low concentration (Fig. 1a). Several workers have reported the involvement of higher concentrations of peroxidase in the decolorization of dyes.[22] It can be evaluated from the data that around 82% decolorization of DB 80 was achieved only in presence of 0.2 mM of HOBT and maximum decolorization (97%) was obtained at 0.6 mM of HOBT which is quite low concentration (Fig. 1b). It was quite related to the earlier study in which maximum decolorization was obtained by turnip peroxidase at 0.6 mM HOBT. [23] While in other studies it has been shown that maximum decorolization was achieved at higher concentration of HOBT which is not good as it also add to the cost of treatment and may also cause toxicity to the environment, if they are released into the water bodies.[24]

Fig. 1c demonstrates that 0.3 mM H_2O_2 was sufficient for the maximum decolorization of the dye which is very low as compared to concentration required by other peroxidases for the decolorization of synthetic dyes.[22, 25] Low concentration of H_2O_2 required by GP may decrease the cost of treatment as the H_2O_2 is an expensive co-substrate.[10] Percent decolorization remained same upto 0.75 mM. A slight decrease started from the concentration greater than 0.75 mM as it was reported that high concentration of H_2O_2 act as an inhibitor of peroxidase as it irreversibly oxidise the ferriheme group of the peroxidase which is essential for its activity.[26]

The optimum pH was found to be 5 for maximum decolorization by GP (Fig. 1d). This data supported by ealier studies in which maximum decolorization by peroxidase from turnip and garlic peroxidase was obtained at pH 5.[27, 28] In earlier studies it was observed that maximum decolorization by peroxidase was obtained at acidic pH such as *Citrus limon* peroxidase at pH 4.5 and horseradish peroxidase at pH 6 which supports the findings.[3, 29] The significant amount of DB 80 was decolorized over a wide range of temperature i.e. from 20–60°C (Fig. 1e). Maximum decolorization was obtained at 40°C. This result was found in agreement with earlier studies in which temperature optima was 40°C.[30]

Effect of time on the decolorization of dyes was also studied and around 88% decolorization was obtained within 15 min (Fig. 1f). The differences in time required for the decolorization of various dyes might be due to structural barrier and electron localization among them.[31] Kinetics studies were also done in order to find out the K_m and V_{max} of the enzyme for the dye. K_m and V_{max} for the enzyme were found to be 27.8 mg/L and 2.09 mg/L/min respectively. V_{max} of GP was found to be very high as compared to earlier studies in which it is comparatively low.[32] In order to confirm the decolorization of dye, UV-visible spectral analysis was performed. Decrease in peak in visible region was due to breakdown of chromophoric groups of the dye (Fig. 3). Similar results were also obtained in previous reports in which there was a diminution in peaks in visible region after decolorization of dyes.[33]

It has been concluded that all the dyes treated by GP in the optimized conditions were removed significantly (Table 1). Some were decolorized to a greater extent, i.e., from 70-96% while only two dyes were decolorized less than 50% (DY 50 46% and AB 210 40%). It was also observed in earlier studies that many synthetic textile dyes were removed significantly by peroxidase.[34] Different mixtures of dyes were also decolorized by GP (Table 2). A considerable amount of all five mixtures were decolorized within 1 h. This will be highly useful for the treatment of colored effluents from textile industries. This is supported by earlier data in which many combinations of dyes were decolorized by peroxidase.[35]

In stirred batch process decolorization of DB 80 was very fast i.e. 98.3% of dye was decolorized in a time period of just 1 h with only 12.5 U of GP (Table 3). The result was far more better than previous one in which only 70-75% decolorization of synthetic dyes were obtained in 4 h with 40 U of peroxidase.[36]

Genotoxic assessment was also done for both DB 80 and its decolorized product (Fig.4, 5). It was found that genotoxicity was decreased to a greater extent after treatment with GP. Therefore, it can be concluded that decolorized product was found less toxic as compared to its parent compound DB 80. This report was in agreement with earlier findings in which textile dyes and textile effluent were reported more genotoxic than their decolorized products. Karimet al.[25] observed the DNA

damage in textile effluent treated lymphocytes by comet assay while after decolorization no DNA damage was found in lymphocytes. Similarly dye decolorized products of mixture of textile dyes treated by bacterial strain Alishewanella sp. KMK6 was less genotoxic than dye mixture as assessed by comet assay.[37]

It is depicted from Table 4 that MI of *Allium cepa* was increased after the treatment of DB 80 which clearly suggests that antiproliferative activity of DB 80 was destroyed by GP. The results also show that DB 80 is highly cytotoxic and putting a strong inhibitory effect on the cell division. Earlier studies reported that dyes present in textile effluents are responsible for decreased mitotic index of *Allium cepa* roots exposed to it.[38] The decrease in prophase index and increase in metaphase and anaphase-telophase index after the treatment of DB 80 might be due to removal of blockage of chfr point (control point between prophase/ metaphase), which is a checkpoint that delays entry into metaphase in response to mitotic stress.[39]

For the investigation of genotoxicity potential of the textile dyes the determination of chromosomal aberrations may be considered as an important and efficient test.[40] It was observed in previous studies that textile dyes were more genotoxicand causes chromosomal aberrations in *Allium cepa* roots.[14, 41] In our study DB 80 was found to be highly genotoxic causing chromosomal aberrations in mitotic cells of *Allium cepa* roots which was not reported earlier (Fig. 6). It can be evaluated from Table 5 that genotoxicity was significantly decreased after treatment of DB 80 by GP. Percentage of total aberrant cells of distilled water and treated DB 80 was 2.3 and 5, respectively which was comparable, suggesting that toxicity was almost diminished by GP.

V. CONCLUSION

This study demonstrated that synthetic dyes released by textile industries were effectively removed by GP in conjunction with redox mediator HOBT. The enzyme was quite active over a wide range of pH and temperatures which makes the GP applicable at industrial level for the decolorization of dyes. H_2O_2, which is an expensive co-substrate, was required in very low concentration for optimum decolorization of dyes. DB 80 was successfully decolorized in a stirred batch process in a very short time period which can make the process feasible for treatment of textile effluents. The enzyme was partially purified from easily available and inexpensive source that can overcome the high cost of purification of enzyme which acts as a barrier for its application in wastewater treatment. Also the DB 80 was significantly detoxified which implies that environment can be free of hazardous dyes by this redox mediated enzyme system.

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