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DECOLORIZATION OF INDUSTRIAL DYES BY AN EXTRA-CELLULAR PEROXIDASE FROM *BACILLUS* SP. F31

Neelam Bansal and Shamsher S Kanwar
Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-171005 India.
Email: Neelam06@gmail.com[1]; Kanwarss2000@yahoo.com[2]

ABSTRACT:
Aim: An extra-cellular peroxidase purified from a bacterial strain *Bacillus* sp. F31 was evaluated for its ability to decolorize 16 different industrial dyes.
Methods and Results: The bacillus sp. F31 peroxidase was purified from culture broth by $(NH_4)_2SO_4$ fractionation, dialysis and anion-exchange chromatography Each of the selected dyes namely Bromophenol Blue (BPB), Reactive Yellow FN2R (RY), Congo Red (CR), Xylidine (XY), Methyl Orange (MO), Rhodamine B (RB), Erichrome Black Y (EB), Bismark Brown R (BBR), Basic Fuchsin (BF), Bismark Brown Y (BBY), Direct Violet 21 (DV), Direct Black 154 (DB), Methylene Blue (MB), Black RL (BRL), Coomassie Brilliant Blue G-250 (CBBG) and Malachite Green (MG) was subjected to treatment with purified peroxidase (0.75 U) at 37°C for 30 min in phosphate citrate buffer (pH 5.2).
Conclusions: Out of 16 different textile dyes the *Bacillus* sp. peroxidase efficiently decolorized 5 dyes out of which 4 triphenyl methane dyes (BF, RB, CBBG and MG) showed decolorization up to 95.5%, 70.8%, 70% and 40%, respectively, while a polymeric heterocyclic dye (MB) showed 66.2% decolorization. These 5 dyes were studied to further enhance their decolorization by purified peroxidase by optimizing different reaction conditions (temperature, time, enzyme concentration, buffer pH, dye concentration and effect of various salt ions).
Significance and Impact of Study: The observed results could be extended to employ *Bacillus* sp. F31 peroxidase for the treatment of industrial effluents containing common textile dyes. This approach will provide an effective application of peroxidase in managing effluents containing dyes.

Keywords: Extra-cellular, decolorization, industrial dyes, optimization

INTRODUCTION:

Industrial dye wastes represent one of the most problematic groups of pollutants that are not easily biodegradable Ong *et al.* (2011). The main consumers of dyes are the textile, plastic, tannery, paper & pulp and electroplating industries (Parshetti *et al.* 2009; Du *et al.* 2011 Chanwun *et al.* 2013, Dhankhar *et al.* 2020). Dyes are also used as additives in petroleum products. In addition to above applications of dyes, a number of dyes and dyestuffs are also used in the food, pharmaceutical and cosmetic industries (Huber and Carre 2012). Based on the chemical structure of the chromophoric group, the dyes are classified as azo, anthraquinone, triarylmethane and phthalocyanine dyes (Mathur and Kumar *et al.* 2013, Tian *et al.* 2016). It is estimated that between 10-20% of dyestuff being used in the dyeing process could be found in wastewater. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds which act as serious environmental pollutants (Singh *et al.* 2010, Okonkwo *et al.* 2021).

In whole world every year the total production of industrial products is less than the production of industrial effluents and due to this the ecological balance is continuously disturbing. The food web/ food chain of

organism also ill effected with the toxic pollutants released from distillery effluents in the environment (Sugano *et al.* 2009; Pal and Vimala, 2012; Ramachandran *et al.* 2013; Amar *et al.* 2014, Jaiswal *et al.* 2016). The presence of dyes or their degraded products in water can also cause human health disorders such as nausea, hemorrhage, ulceration of skin and mucous membranes. The presence of such compounds also resulted into severe damage to the kidney, reproductive system, liver, brain and central nervous system (Husain, 2010). Synthetic dyes represent a broad and heterogeneous class of durable pollutants that are released in large amounts by the textile industry. Color durability is the most important goal of dying process, so textile dyes of all chemical classes are commonly highly resistant to both chemical and physical degradation (Zucca *et al.* 2012; Celebi *et al.* 2013, Alba *et al.* 2019).

Most experiments on degradation of dyestuffs by microbial peroxidase have been carried out using either whole culture, crude or purified preparations of the peroxidase. Most of the peroxidases catalyses asymmetric cleavage of bonds present in the dyes to decolorize or degrade them (Dawkar *et al.* 2009; Shinya *et al.* 2010; Lin-Na *et al.* 2011; Renugadevi *et al.* 2011; Saladino *et al.* 2013, Chao and Li, 2015). Although currently available methods such as chemical oxidation, reverse osmosis, adsorption, incineration, photo-catalysis or ozonation to dye removal are highly efficient but they suffer from some disadvantages (Telke *et al.* 2010). The limitations include high cost, limited applicability, high energy input and usually these treatments may result in the production of toxic by-products. Due to the inherent drawbacks of physical, chemical and photo-chemical approaches (Singh *et al.* 2010) the use of biological methods for the decolourization and detoxification of textile wastewaters dyes has received attention as a more cost effective alternative, eco-friendly nature as well as the property of producing a less amount of sludge (Franciscon *et al.* 2012, Xing *et al.*, 2018).

A number of studies on the degradation of industrial dyes by bacteria and fungi have indicated the involvement of extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, laccase, cellobiose dehydrogenase and other H_2O_2-producing enzymes (Joshi *et al.* 2010; Kurade *et al.* 2011; Marco-Urrea and Reddy, 2012; Shah *et al.* 2013). The biodegradation of dyes by ligninolytic enzyme like peroxidase system offers an advantage over other processes because of their ability to completely mineralize various dyes to CO_2 and H_2O (Faraco *et al.* 2007; Krishnaveni *et al.* 2011, Durdic *et al.* 2021).

Peroxidases are highly non-specific and are able to transform or mineralize organo-pollutants as well as decolorize various dyes (Chaieb *et al.* 2009; Deivasigamani *et al.* 2011, Lauber *et al.* 2017, Morsy *et al.* 2020). Therefore, the aim of the present investigation was to study the biodegradation of various industrial dyes by an extra-cellular peroxidase produced from a bacterial isolate *Bacillus* sp. F31 which was isolated from crude oil-polluted soil. Further, the purified bacterial peroxidase showed different specificities and efficiencies toward different industrial dyes.

1. MATERIALS AND METHODS

1.1 Organism

The peroxidase-producing bacterial strain *Bacillus* sp. F31 was originally isolated

from soil samples of crude oil-polluted soil from District Mandi (Himachal Pradesh, India).

1.2 Preparation of crude enzyme

The seed culture of *Bacillus* sp. F31 was raised at 37°C (120 rpm) for 24 h. The seed culture was transferred (10%, v/v) to 50 ml of production broth [containing yeast extract (0.2%), beef extract (0.1%), glucose (1.4 %), peptone (0.5%), NaCl (0.5), H_2O_2 (0.06%; v/v) with final pH of 7.5] followed by incubated for 48 h under shaking conditions at 120 rpm at 37°C. Thereafter, the incubated broth was harvested by centrifugation (10,000 X g for 15 min at 4°C; SIGMA 3K30, Germany) and the cell-free broth was termed as crude peroxidase.

1.3 Protein and peroxidase assay

Protein concentration was measured by a standard method (Bradford, 1976) using Bovine serum albumin as a standard. The assay of peroxidase in the broth or purified enzyme fraction was done by a colorimetric method using *o*-phenylenediamine (OPD) as a chromogen and H_2O_2 as a substrate. The assay mixture consisted of 550 µl of freshly prepared H_2O_2 (0.15%, v/v) in 10 ml of 0.1 M Phosphate-citrate buffer, pH 5.2, 5.0 mg of OPD and 50 µl of purified enzyme. The reaction mixture was incubated at 37°C for 10 min in a dry heating block (Boviard *et al.* 1982; Hamilton *et al.* 1999) and thereafter 1 N HCl was added to stop the enzymatic reaction. The A_{492} values were recorded and activity of the peroxidase was determined.

Enzyme unit of peroxidase

One unit (U) of peroxidase was defined as the amount of enzyme needed to convert 1.0 µM of chromogenic substrate (OPD) to its product (2, 3 diamino-phenazine) per min at pH 5.2 and temperature 37°C.

1.4 Purification

An extracellular bacterial peroxidase produced by bacterial isolate *Bacillus* sp. F31 was purified by $(NH_4)_2SO_4$ fractionation, dialysis and anion-exchange chromatography (DEAE-cellulose column chromatography). The molecular mass of purified peroxidase was determined by SDS-PAGE.

1.5 Application of purified bacterial peroxidase of *Bacillus* sp. F31 in decolourization of textile dyes

Screening of industrial dyes for decolorization by purified peroxidase

To test the ability purified peroxidase to decolorize selected industrial dyes, a total of 16 different dyes namely, BPB, RY, CR, XY, MO, RB, EB, BBR, BF, BBY, DV, DB, MB, BRL, CBBG and MG were studied using dye decolorization assay mixture containing 700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2, 50 µl of different dyes (20 µM) and 0.75 U purified peroxidase to make the final volume 1 ml in different cuvettes. After incubation at 37°C for 30 min, decolorization of the test dye was assayed by measuring the absorbance at the respective wavelength (λmax) of the dye and decolorization (%) was determined as follows:

Decolorization % =

$$\frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

Optimization of reaction conditions for dye decolourization by purified peroxidase of *Bacillus* sp. F31

Out of 16 dyes tested for decolorization, only 5 dyes (BF, RB, MB, CBBG and MG) that were efficiently decolorized by bacterial peroxidase were selected for further studies. The purified peroxidase was used to evaluate the effect of temperature, reaction time, enzyme quantity, buffer system pH, effect of dye concentration, effect of salt-ions on degradation of selected dyes and optimized conditions for dye degradation were ascertained.

1.5.1 Effect of temperature on dye decolourization

In order to determine optimum temperature for selected dyes (BF, RB, MB, CBBG and MG), the degradation reaction facilitated by peroxidase [1 ml reaction mixture containing 700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2, 0.75 U purified peroxidase and 50 µl (20 µM) of different dyes] was carried out at few selected temperatures (25, 30, 35, 37, 40 and 45°C) for 30 min in Eppendorf tubes in a dry heating block for each dye, separately. After incubation, decolourization was assayed by measuring the absorbance at the respective wavelength of the dyes, and decolourization % was determined and analysed.

1.5.2 Effect of reaction time on dye decolorization

To determine the optimum reaction time for degradation of selected dyes (BF, RB and MB, CBBG, MG) the time of dye degradation assay [1 ml reaction mixture containing 700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2, 0.75 U purified peroxidase and 50 µl (20 µM) of different dyes] was varied from 0 to 40 min for each dye separately. After incubation decolorization (%) of each dye was calculated after incubation at 30°C for RB, 35°C for MB; 40°C for BF, CBBG and MG.

1.5.3 Effect of biocatalyst(enzyme) concentration on dye decolourization

The concentration purified peroxidase was varied from 0.70 U to 1.1 U in 1 ml final reaction volume to perform the dye degradation assay [1 ml reaction mixture containing 700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2 and 50 µl (20 µM) of selected dye]. The decolorization (%) of different dyes was calculated after incubation of 35 min at 35°C for BF; 45 min at 35°C for MB, 40 min at 40°C for RB; 40 min at 30°C for CBBG and 40 min at 40°C for MG.

1.5.4 Effect of buffer system pH on dye decolourization

The effect of reaction buffer pH on the dye decolouration of selected dye by purified peroxidase was studied at different buffer pH values (3-7) of 0.1 M phosphate citrate buffer in (1 ml; containing 15 µl H_2O_2, 50 µl (20 µM) of selected dye). The decolorization (%) of the dyes was calculated after incubation at 35°C for BF after 35 min, at 40°C for RB after 40 min, at 35°C for MB after 45 min, at 30°C for CBBG after 40 min and for MG after 40 min at 40°C, respectively.

1.5.5 Effect of dye concentration on its decolourization

The concentration of each dye was varied from 100 to 1000 mg/l [in dye decolouration assay mixture containing 700 µl (0.1 M) phosphate citrate buffer, 15 µl H_2O_2, 50 µl of different dyes and optimized concentration purified peroxidase for each dye]. The decolorization assay was carried out at respective optimized temperature, pH and

time for each of the dyes and decolorization (%) was determined thereof.

1.5.6 Effect of H_2O_2 concentration on dye decolorization

The concentration of H_2O_2 was varied from 0.25 to 3.5 mM in the mixture [containing 700 µl (0.1 M) phosphate citrate buffer, 50 µl (20 µM) of selected dyes and optimized concentration of purified peroxidase]. The decolorization assay was carried out at respective optimized temperature, pH and time for each of the dye and decolorization (%) was determined in each case.

1.5.7 Effect of salt-ions and inhibitors on dye decolorization

The decolorization (%) of BF, RB, MB, CBBG and MG with purified peroxidase was determined in the presence of 1 mM (w/v) of selected salt ions and inhibitors (Li^{+3}, Zn^{+2}, Mg^{+2}, K^{+2}, Na^{+}, Hg^{+2}, Mn^{+2}, Ca^{+2}, Cu^{+2}, Fe^{+2}, EDTA, SDS, sodium azide and DTT) at optimized conditions. The mixture of enzyme and metal ion/ inhibitors in ratio 1: 1 was pre-incubated for 30 minutes at 37°C in a dry heating bath. Thereafter, the pre-incubated peroxidase was separately incubated with each of the selected dye in decolorization assay. The decolorization assay was carried out at respective optimized temperature, pH and time for each of the dye and decolorization (%) was determined in each case.

2. RESULTS:

2.1 Purification

The purification of peroxidase from *Bacillus* sp. F31 was done by ammonium sulphate precipitation, dialysis and anion exchange chromatography (DEAE-cellulose). The protein analysed by the SDS-PAGE and the native-PAGE resulted in a single band of approximately 37 kDa and 95 kDa, respectively. The enzyme was purified up to 14.6-fold with a yield of 12.6 %.

2.2 Applications of purified bacterial peroxidase of *Bacillus* sp. F31 in decolourization of textile dyes

Screening of industrial dyes for decolorization by purified peroxidase

To test the ability of bacterial peroxidase to decolorize a few selected industrial dyes namely BPB, RY, CR, XY, MO, RB, EB, BBR, BF, BBY, DV, DB, MB, BRL, CBBG and MG, each of these dyes was subjected to treatment with purified peroxidase (0.75 U) at 37°C for 30 min in phosphate citrate buffer (pH 5.2). Out of these 16 textile dyes, the purified peroxidase efficiently decolorized 5 dyes out of which 4 belonging to triphenyl methane group (BF, RB, CBBG and MG) showed decolorization up to (95.5, 70.8, 70.0 and 40.0%, respectively) while a polymeric heterocyclic dye (MB) showed 66.2% decolorization (Table 1 and Table 2). These 5 dyes were selected to further enhance their decolorization by purified peroxidase by optimizing reaction conditions (temperature, time, enzyme concentration, buffer pH, dye concentration and effect of various salt ions).

Optimization of reaction conditions for decolorization of selected dyes by purified peroxidase of *Bacillus* sp. F31

2.2.1 Effect of temperature on dye decolorization

The temperature of reaction system was varied from 30 to 45°C for decolorization of BF, RB, MB, CBBG and MG separately in the reaction mixture (1 ml) containing 0.75 U of purified peroxidase. The optimum temperature

for each of these dye with purified peroxidase was 30°C for RB (72.1%), 35°C for MB (82.0%), 40°C for BF (92.1%), CBBG (90.2%) and MG (65.2%), respectively (Fig. 1).

2.2.2 Effect of reaction time on dye decolorization by purified peroxidase

The reaction time of dye decolorization for each of the selected dyes (BF, RB, MB, CBBG and MG) was varied from 0 to 45 min. The maximum decolorization by purified peroxidase was observed between 30-45 min for MB (82.1%, 30 min) at 35°C, BF (96.1%, 35 min) at 40°C, RB (76.2%, 40 min) at 30°C, CBBG (90.0%, 40 min) and MG (78.3%, 40 min) at 40°C, respectively (Fig. 2).

2.2.3 Effect of biocatalyst concentration on dye decolorization

The enzyme concentration used in dye decolorization assay of 5 selected dyes (BF, RB, MB, CBBG and MG) was varied from 0.77 to 1.05 U for purified peroxidase in the 1 ml final volume of reaction mixture. The maximum decolorization was observed with 0.94 U of purified peroxidase for BF (95.1%) at 40°C in 35 min; 1.05 U for RB (82.2%) at 35°C in 40 min, MB (85.1%) at 35°C in 30 min and MG (78.2%) at 35°C in 40 min, and 1.01 U for CBBG (92.1%) at 40°C in 40 min, respectively (Fig. 3).

2.2.4 Effect of buffer pH on dye decolorization

In order to determine optimum pH of the assay buffer system for efficient decolorization of each dye (BF, RB, MB, CBBG and MG) the studies with selected dyes were performed with purified peroxidase by varying buffer pH (phosphate citrate buffer) from 3-7. The optimum buffer pH for each of the dyes was pH 5.0 for RB (81.2% at 35°C for 40 min with 1.05 U of purified peroxidase); pH 5.5 for BF (96.1% at 40°C for 35 min with 0.94 U peroxidase) and also for MB (83.5% at 35°C for 30 min with 1.05 U of peroxidase) and MG (78.3% at 40°C for 40 min with 1.05 U of peroxidase); pH 6.0 for CBBG (92.2% at 40°C for 40 min with 1.01 U of peroxidase), respectively (Fig. 4).

2.2.5 Effect of dye concentration on its decolorization

The maximum decolorization of BF was found to be 97.1% at concentration 800 mg/l at 40°C in 35 min at pH 5.5, for RB (86.2%) at 600 mg/l at 30°C in 40 min at pH 5.0, MB (84.2%) at 35°C in 30 min at pH 5.5 and MG (78.1%) at 400 mg/l at 40°C in 40 min at pH 5.5 and CBBG (92.1%) at 40°C in 40 min with 200 mg/l at pH 6.0 (Fig. 5).

2.2.6 Effect of H_2O_2 (substrate) concentration on dye decolorization

When the concentration of H_2O_2 was varied from 0.25 to 3.5 mM in dye decolorization assay, the optimized concentration of H_2O_2 for decolorization of all selected dyes (BF, RB, MB, CBBG and MG) using purified peroxidase was found between 1 to 1.5 mM with a decolorization of 97.1% for BF at 40°C in 35 min, 86.2% for RB at 30°C in 40 min, 84.1% for MB at 35°C in 30 min, 92.3% for CBBG at 40°C in 40 min and 78.1% for MG at 40°C in 40 min, respectively (Fig. 6).

2.2.7 Effect of salt-ions and inhibitors on dye decolorization

The decolorization (%) of BF, RB, MB, CBBG and MG with purified peroxidase was also determined in the presence of selected salt ions and inhibitors under optimized conditions. The results indicated that the decolorization of all the 5 dyes by purified peroxidase was inhibited by the presence of

Hg^{+2}, EDTA, sodiun azide, DTT and SDS. However, the decolorization was found to be slightly stimulated by purified peroxidase in the presence of Zn^{+2} (BF 100.5%, RB 101.2%, MB 101.1%, CBBG 101.1% and MG 101.2%), Mg^{+2} (BF 101.6%, RB 101.2%, MB 100.5%, CBBG 101.6% and MG 102.5%) and Mn^{+2} (RB 100.6% and MG 100.2%), respectively (Table 3).

DISCUSSION:

The potential use of an extra-cellular peroxidase purified from a bacterial isolate *Bacillus* sp. F31 in the decolorization of the few common textile dyes was studied. The purified peroxidase appeared better in achieving efficient decolourization of BF, RB, MB, CBBG and MG. Peroxidase from *Bacillus* sp. F31 decolorize four triphenyl methane dyes and one polymeric heterocyclic efficiently so it could be stated that the peroxidase of *Bacillus* sp. F31 has higher affinity for triphenyl methane dyes as substrates. In a previous study, the peroxidase from *Hevea brasiliensis* was able to decolorize triphenyl methane dye efficiently as compare to other groups of synthetic dyes Chanwun *et al.* (2013). The decolourization of dyes by an enzyme depends upon many factors such as their chemical structure, molecular weight (*M*r), redox potential, molecular weight, complexity of side chains and most importantly the binding site of enzyme (Azmi *et al.* 1998). The BF having a simple structure with small functional groups (NH_2; side chains) and a relatively lower *M*r (337.8 g/mol) was efficiently decolourized as compare to other dyes bearing more complex side chains. The side chains often account for stearic hindrance in binding to the enzyme. This can be possibly the reason for greater decolourization of the BF dye by purified peroxidase of *Bacillus* sp. F31.

In the previous studies many type of peroxidases purified from various sources were found to decolorize different industrial dyes (Shin *et al.* 2005; Hong *et al.* 2012; Nashwa *et al.* 2013., Amar *et al.* 2014, Jaiswal *et al.* 2016; Durdic *et al.* 2021). The MnP purified from *Dichomitus squalens* was also able to decolorize selected azo and anthraquinone dyes (Susula *et al.* 2008). The manganese-independent peroxidase sourced from *Auricularia uricular-judae* was found to be stable in decolorization of the high-redox potential dyes like Reactive Blue 5 and Reactive Black 5 (Liers *et al.* 2010). *Ganoderma cupreum* AG-1 isolated from the decayed wood was evaluated for its ability to decolorize azo dyes (Gahlout *et al.* 2012). The optimum temperature for decolouration of the dye with purified peroxidase of *Bacillus* sp. F31 was 30°C for RB (72.1%), 35°C (82.0%) for MB; and 40°C for BF (92.1%), CBBG (90.2%) and MG (65.2%), respectively. The recorded data showed that purified peroxidase performed efficient decolourization of chosen common textile dyes at 30-40°C. In previous studies for the decolourization of MG, a constant temperature of 25± 0.5°C (Satapathy *et al.* 2011) and 30°C for the peroxidase of a fungal strain *Cunninghamella elegans* was reported (Roushdy *et al.*, 2011). The peroxidase from *Pleurotus ostreatus* also decolorized triphenyl methane dyes (BPB and MB) at 25°C (Shin *et al.* 1998). BF was 93% decolorized at 30°C by a peroxidase from *Aeromonas hydrophila* (Ogugbue *et al.* 2012). The optimum temperature for decolorization of different dyes by peroxidase from *Trametes versicolor* was found to be 30°C (Celebi *et al.* 2013).

When the effect of reaction/ treatment time on the decolourization of five

selected textile dyes was evaluated, the purified peroxidase of *Bacillus* sp. F31 provided optimal decolourization for MB (82.1%, 30 min) at 35°C, BF (96.1%, 35 min) at 40°C, RB (76.2%, 40 min) at 30°C, CBBG (90.0%, 40 min) and MG (78.3%, 40 min) at 40°C, respectively. Thus the purified biocatalyst efficiently performed decolourization of selected dyes between 35-45 min at the chosen optimized temperature. The decolourization of the dye increased with the extension of time but little increase in decolourization was noticed after 40 min for each dye. In another study, the optimum time for MB decolourization was also found 40 min (Satapathy *et al.* 2011).

The removal of an organic pollutant is dependent on the amount of catalyst added and the contact time. Thus there is often an optimum relationship between the concentration of enzyme and substrate for achieving maximum activity. The enzyme concentration used in dye decolourization assay reaction of five selected dyes (BF, RB, MB, CBBG and MG) in the present study was varied from 0.77 to 1.05 U of purified peroxidase of bacillus sp. F31 in the 1 ml final volume of the reaction mixture. The maximum decolourization with purified enzyme was observed with 0.94 U of peroxidase for BF (95.1%) at 40°C in 35 min, 1.05 U for RB (82.2%) at 35°C in 40 min, MB (85.1%) at 35°C in 30 min and MG (78.2%) at 35°C in 40 min and 1.01 U for CBBG (92.1%) at 40°C in 40 min, respectively. In a previous study, an increase in the Soya bean peroxidase dose from 10 to 80 U/ml resulted in a gradual increase in the dye removal (16-64%) that appeared to be levelling off at 80 U/ml (Kalsoom *et al.* 2013).

The intact enzyme may contain both positively and negatively charged groups at any given pH. Such ionizable groups are often part of the active site (Gomare *et al.* 2008). Variation in the pH of the medium can result in changes in both the ionic forms of the active site and the activity of enzyme and consequently, the reaction rate (Hossain and Anantharaman 2006). The optimum buffer pH for each of the dyes using purified peroxidase of *Bacillus* sp. F31 was pH 5.0 for RB (81.2% at 35°C for 40 min with 1.05 U), pH 5.5 for BF (96.1% at 40°C for 35 min with 0.94 U) and also pH 5.5 for MB (83.5% at 40°C for 30 min with 1.1 U) and MG (78.3 % at 40°C for 40 min with 1.05 U), pH 6.0 for CBBG (92.2% at 40°C for 40 min with 1.01 U), respectively. All the selected dyes were thus effectively decolorized in pH range 5.0-6.0 and at the higher values, the dyes showed poor degradation. This observation seems similar to what other workers have reported (Marchis *et al.* 2011; Zhang *et al.* 2012 Tian *et al.* 2016) and underscores the usefulness of this enzyme to degrade industrial effluents which might be very acidic in nature. In another study, it was observed that most of the dyes including MG decolorized at strong to moderate acidic pH (pH 2-6) values (Zucca *et al.* 2012). The reactions steps of the catalytic cycle of peroxidase are pH dependent and appeared to work best under acidic conditions. In the initial step, the formation of compound I is favoured by the presence of a network of hydrogen bonds between the Fe-heme/ H_2O_2 adduct and the distal histidine and arginine side chains, whereas, in the subsequent steps, the substrate oxidation depends on its protonation state (Kalsoom *et al.* 2013; Alba *et al.* 2019).

In the present study, the maximum decolorization of BF using bacillus sp. F31 peroxidase was found to be 97.1% at concentration 800 mg/l at 40°C in 35 min at pH 5.5, for RB (86.2%) at 600 mg/l at 30°C in 40 min at pH 5.0, MB (84.2%) at 35°C in 30 min at pH 5.5 and MG (78.1%) at 400 mg/l at 40°C in 40 min at pH 5.5 and CBBG (92.1%) at 40°C in 40 min with 200 mg/l at pH 6.0. It appeared quite possibly due to the reason that if the concentration of enzyme is kept constant and the substrate concentration is gradually increased the reaction would continue until it reaches maximum. After obtaining the equilibrium state any further addition of the substrate would not change the rate of reaction. The effect of dye concentration in reaction mixture is an important consideration for its field or application. The dye concentration in effluent from textile printing house is approximately 200-800 mg/l (Zhao and Hardin 2007). According to a recent study, decolourization efficiency decreased with increasing dye concentration and a marked inhibition was exhibited when the dye (Remazol Brilliant Blue R) concentrations were above 100 mg/l (Silva *et al.* 2013). The FTIR spectroscopy, NMR and GC-MS of several dye degradation products using purified peroxidase by *Bacillus cereus*, the results confirmed that decolorization was due to breakdown of dyes into unknown products (Fetyan *et al.* 2013).

The decolourization of BF, RB, MB, CBBG and MG with purified peroxidase of *Bacillus* sp. F31 was also determined in the presence of selected salt ions and inhibitors under optimized conditions of decolouration. The results indicated that the decolourization of all the five dyes by purified peroxidase was inhibited in the presence of Hg^{+2}, EDTA, sodiun azide, DTT and SDS. However, the decolourization was found to be slightly stimulated for purified peroxidase in the presence Zn^{+2}, Mg^{+2} and Mn^{+2}. Some bivalent metal ions such as Mg^{+2}, Zn^{+2} and Co^{+2} might enhanced the peroxidase activity so the use of such ions could be considered in dye decolourization experiments as additives for efficient dye decolourization (Dawkar *et al.* 2009; Irshad and Asgher 2011; Si and Cui, 2013). In another study, Mg^{+2} and Mn^{+2} (1 mM) ions were observed to significantly enhance the decolourization of MG by peroxidase from *Pseudomonas* sp. (Du *et al.* 2011).

H_2O_2 reacts with the purified *Bacillus* sp. F31 peroxidase to oxidize the native enzyme to form an enzyme intermediate, which easily accepts an aromatic compound to carry out its oxidation to a free radical form. In this regard, experiments were done wherein the decolouration of the selected (BF, RB, MB, CBBG and MG) textile dyes was measured as a function of H_2O_2 concentration, while keeping the other reaction parameters constant. When the concentration of H_2O_2 was varied from 0.25 to 3.5 mM in dye-decolourization assay, the optimized concentration of H_2O_2 for decolourization of these dyes using purified peroxidase was found to be between 1 to 1.5 mM with a decolourization of 97.1% for BF at 40°C in 35 min, 86.2% for RB at 30°C in 40 min, 84.1% for MB at 35°C in 30 min, 92.3% for CBBG at 40°C in 40 min and 78.1% for MG at 40°C in 40 min, respectively. H_2O_2 alone or in conjunction with other materials is often used for oxidation and degradation/ decolourization of many harmful organic compounds including dyes. The addition of a small amount of catalyst to a system containing H_2O_2 may lead to the generation of

free radicals like •OH with a reasonably high reduction potential (2.3 eV) that facilitates faster degradation of many organic compounds. On the contrary, higher H_2O_2 was detrimental to the process, most likely due to the damage to the enzyme being a protein itself. Thus it becomes pertinent to optimize the H_2O_2 concentrations in the enzyme-based dye degradation approaches (Zhang *et al.* 2012). For the decolourization of MB by HRP, 0.15 mM H_2O_2 concentration in the reaction mixture was found to be the optimum (Satapathy *et al.* 2011). In case of dyes degradation with Soya bean peroxidase, H_2O_2 concentration led to an increased dye decolourization. However, after reaching the maximum dye decolourization with 64 µM H_2O_2, any further increase in H_2O_2 did not cause any additional effect (Kalsoom *et al.* 2012).

In the previous studies many type of peroxidases purified from various sources were reported to decolorize different industrial dyes (Shin *et al.* 2005; Hong *et al.* 2012; Celebi *et al.* 2013, Salvachua *et al.* 2013; Chao and Li, 2015;). The MnP purified from *Dichomitus squalens* was also able to decolorize selected azo and anthraquinone dyes (Susula *et al.* 2008). BF, RB and MG are triphenyl methane type of basic dyes used in textile, pharmaceutical and chemical industries; while RB used extensively in textile industries for dying nylon, wool, silk, and cotton. Sometimes it is also used in medicine, paper, leather, food, cosmetics industries. On other hand, MB is a polymeric/ heterocyclic dye used in textile dyeing, pharmaceuticals, paper industry and also as a biological strain. These all dyes are discharged in industrial effluents pose serious threat to human health and environment. The great potential of peroxidase produced by *Bacillus* sp. F31 might be adopted as an effective tool to efficiently decolorize most of the common textile dyes. A thermostable peroxidase from *Bacillus stearothermophilus* and peroxidase sourced from *Auricularia auricula-judae* has been found to be stable in decolourization of the high-redox potential dyes such as Reactive Blue 5 and Reactive Black 5 (Loprasert *et al.* 1988; Liers *et al.* 2010). *Ganoderma cupreum* AG-1 recently isolated from the decayed wood was evaluated for its ability to decolorize azo dyes Gahlout *et al.* (2013). Present results suggested that the purified peroxidase from *Bacillus* sp. F31 was effective in discolouration of many common textile dyes. In some recent studies, bacterial peroxidase(s) have been found to be efficient biological decolourization tools (Zucca *et al.* 2012; Saladino *et al.* 2013, Lauber *et al.* 2017, Morsy *et al.* 2020).

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Legends to the tables

Table 1 Screening of dyes for decolourization by purified peroxidase

Table 2 Efficient decolorization of dyes by purified peroxidase of *Bacillus* sp. F 31

Table 3 Effect of metal ions and inhibitors on decolourization of dyes

Table1 Screening of dyes for decolourization by purified peroxidase

S. No.	Name of dye (1 mM)	Type of dye	Dye decolourization (%)
1.	BPB	**Triphenyl methane**	3.0
2.	RY		5.0
3.	CR	Azo	-
4.	XY	*Dimethylaniline*	-
5.	MO	Azo	3.0
6.	**RB**	**Triphenyl methane**	**70.8**
7.	EB	Azo	-
8.	BBR	Diazo	5.0
9.	**BF**	**Basic dye**	**95.5**
10.	BBY	Diazo	7.0
11.	DV	Azo	-
12.	DB	Azo	8.0
13.	**MB**	**Polymeric heterocyclic**	**66.2**
14.	BRL	Azo	4.0
15.	**CBBG**	**Triphenyl methane**	**70.0**
16.	**MG**	**Triphenyl methane**	**40.0**

Table 2 Efficient decolorization of dyes by purified peroxidase of *Bacillus* sp. F31

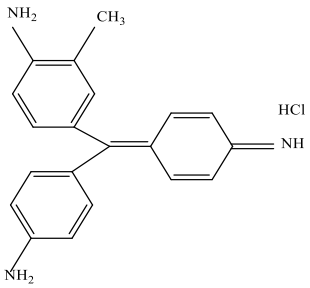
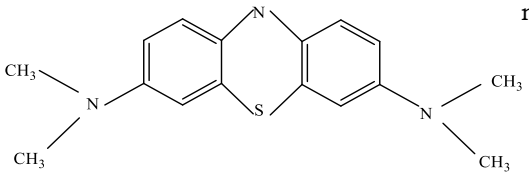
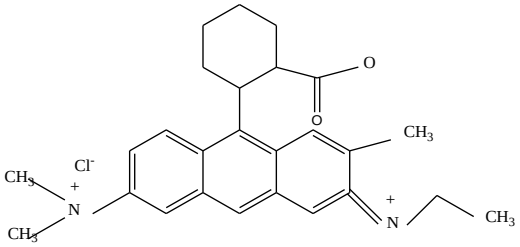
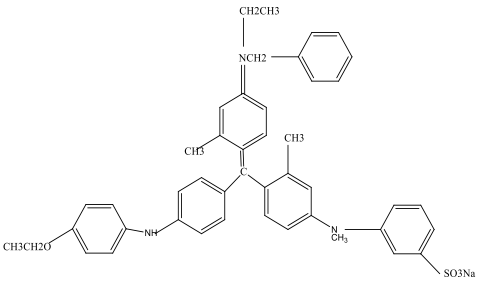
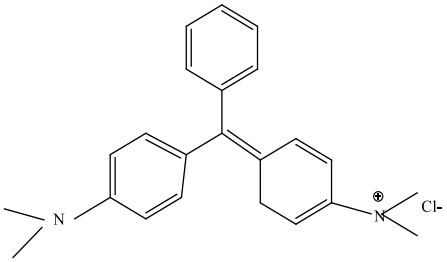
Dye	Structure of dye/ λ_{max}/ Molecular weight (g/mol)	Type of dye	Decolorizatin (%)
BF	 $\lambda_{max=545nm}$, molecular weight= 337.85	Triphenyl methane dye	95.5
MB	 $\lambda_{max=664nm}$, molecular weight= 319.85	Triphenyl methane dye	66.2
RB	 $\lambda_{max=555nm}$, molecular weight= 479.01	Polymeric/heterocyclic dye	70.8
CBBG	 $\lambda_{max=610\ nm}$, molecular weight =854.02	Triphenyl methane dye	70.0
MG	 $\lambda_{max=550\ nm}$, molecular weight= 364	Triphenyl methane dye	40.0

Table 3. Effect of metal ions and inhibitors on decolourization of dyes.

Metal ion/ Inhibitor (1 mM)	**Relative decolourization (%) at stated λ_{max}**				
	BF (A_{545})	RB (A_{555})	MB (A_{664})	CBBG (A_{610})	MG (A_{550})
None	100.0	100.0	100.0	100.0	100.0
Li^{+3}	97.8	88.4	97.6	98.5	95.0
Zn^{+2}	**100.5**	**101.2**	**101.1**	**101.1**	**101.2**
Mg^{+2}	**101.6**	**101.2**	**100.5**	**101.6**	**102.5**
K^{+2}	98.9	98.7	98.8	95.5	92.5
Na^{+}	97.8	Temperature (°C)		93.3	87.5
Hg^{+2}	63.8	62.8	47.0	38.8	31.2
Ca^{+2}	94.6	98.7	96.4	94.4	88.7
Cu^{+2}	96.8	96.1	97.6	97.7	93.7
Fe^{+2}	95.7	93.5	98.8	96.6	96.2
Mn^{+2}	**100.0**	**100.6**	99.4	99.3	**100.2**
EDTA	88.9	80.9	78.9	74.5	72.0
SDS	62.5	56.5	60.7	58.4	54.2
Sodiun azide	45.8	42.3	35.7	50.4	41.5
DTT	58.9	64.5	62.1	60.4	67.0