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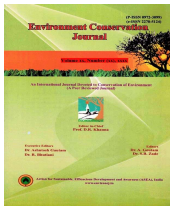

Biodegradation of harmful industrial dyes by an extra-cellular bacterial peroxidase

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ABSTRACT

Nowadays the treatment of environmental pollutants such as synthetic dyes (used in multiple industries such as paper, textile, food, plastic and pharmaceutical) has received much attention, especially for biotechnological treatments using both native and artificial enzymes. In this context, many enzymes have been reported to efficiently perform dye degradation. Peroxidase is one such enzyme, which causes dye degradation either by precipitation of chemical structure of aromatic dyes or by opening up their aromatic ring structure. In the present study an extra-cellular peroxidase extracted from a bacterial strain *Bacillus* sp. F31 JX984444.1 was tested for its capability to decolorize 16 different dyes used in various industries. Out of 16 different textile dyes the *Bacillus* sp. peroxidase efficiently decolorized 5 dyes out of which 4 triphenyl methane dyes (Basic Fuchsin (BF), Rhodamine B (RB), Coomassie Brilliant Blue (CBBG) and Malachite Green (MG) showed decolorization up to 95.5%, 70.8%, 70% and 40%, respectively, while a polymeric heterocyclic dye Methylene Blue (MB) showed 66.2% decolorization. These 5 dyes were studied to further enhance their decolorization by peroxidase after purification by optimizing different reaction conditions (temperature, time, enzyme concentration, buffer pH, dye concentration and effect of various salt ions, H_2O_2 concentration). This study indicates that the extracellular peroxidase (purified) from *Bacillus* sp. can be used as a useful tool for the treatment (degradation/decolorization) of industrial effluents contaminated with harmful industrial dyes.

Introduction

Industrial pollution refers especially to any contamination caused by industrial activities or industrial waste. Different industrial activities, whether it is the extraction of raw material, processing, manufacturing, or waste disposal, leads to environmental degradation. Industrial pollution is a big issue because, most harmful types of pollutants are produced by industries, making it a significant form of pollution on the planet. The effects of industrial pollution are vast, causing contamination, by release of toxic wastes into water, soil, air, and it is the cause of some of the most significant environmental disasters of all time. Water constitutes more than half of our planet, and it is getting polluted day by day due to different causes including human activities, one of the most limelight is industrial chemicals and wastes. The colored wastewater from various industries is the main cause of water pollution. Effects of industrial water pollution are very harmful to animals, plants, human beings as well as entire environment (Ong *et al*., 2011; Hynes *et al.*, 2020; Arif, *et. al*., 2020). The ecological balance is continuously disturbing in whole world every year because, the production of industrial effluents is more than the production of industrial products. The food web/ food chain of organism also severely affected with the toxic pollutants released from industrial effluents in the environment (Sugano *et al*., 2009; Pal and Vimala, 2012; Ramachandran *et al*., 2013; Telke *et al*.,

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2015; Pandey *et al*., 2016; Lellis *et al*., 2019; Verma *et al*., 2021). Therefore, their proper discharge and economical treatment of industrial waste is a matter of great concern.

The various dyes used by several industries are the main cause of colored wastewater and the most problematic groups of pollutants. Harmful synthetic dyes are mostly used in textile, refining, oil, plastic, tannery, paper/pulp, electroplating and cosmetics production units, in the food and pharmaceutical industries (Huber, P., & Carré, 2012; Chanwun *et al*., 2013; Guerra *et al*., 2018; Dhankhar *et al*., 2020). Synthetic dyes used in the textile industry represent a class of highly durable environmental pollutants and non-biodegradable, that are released in large amounts in the rivers and other nearby water reservoirs. These dyes are highly resistant to chemical and physical decomposition methods because colour durability is the most important goal of dying process (Zucca *et al*., 2012; Celebi *et al*., 2013; Blánquez *et al*., 2019). Industrial dyes or their degraded products drown in various sources of water cause many human health disorders such as skin diseases, ulcer, kidney damage, reproductive system diseases, disorders of liver, brain and nervous system (Husain, 2010). Dyes are characterized in specific groups on the basis of chemical structure of the chromophoric groups (Mathur and Kumar *et al*., 2013; Tian *et al*., 2016). Wastewater from various industries is estimated to contain approximately 15-25% of dyestuff that is used in the dyeing process, many of these dyes are recalcitrant compounds that is highly stable to heat cold, light and microbial attack, making them serious pollutants to damage environment (Singh *et al*., 2010). Most experiments on degradation of dyestuffs by microbial peroxidase have been carried out using either whole culture, crude or purified preparations of the peroxidase. Many kids of peroxidases decolorize or degrade the dyes by asymmetric cleavage of bonds present in chromatophoric groups in the dyes (Dawkar *et al*., 2009; Fujihara *et al.,* 2010; Du *et al*., 2011; Renugadevi *et al*., 2011; Saladino *et al*., 2013; Chen & Li, 2016; Ledakowicz & Paździor, 2021). All the currently available techniques for dye removal, such as chemical oxidation, incineration, photo-catalysis or ozonation, reverse osmosis, adsorption are highly efficient but with some disadvantages or limitations (Telke *et al*. 2010). All these treatments may result in the production of toxic by-products, these are highly costly, limited applicability, high energy input etc. Due to the inherent drawbacks of physical, chemical and photo-chemical approaches (Singh *et al*., 2010) the use of biocatalytic or biological methods for the degradation/ decolorization and detoxification of wastewaters dyes has proved as a low cast alternative, nature friendly as well as the property of producing a less quantity of by-products (Franciscon *et al*., 2012; Qin *et al*., 2018).

A number of extracellular oxidative enzymes extracted from different sources such as bacteria, fungi, yeast as well as plants like laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, cellobiose dehydrogenase and other oxidoreductases enzymes have been reported on the degradation of industrial dyes (Alam *et al*, 2010; Joshi *et al.,* 2010; Kurade *et al*., 2011; Marco-Urrea and Reddy, 2012; Qin *et al*., 2018; Chang *et al*., 2021). The oxidoreductase enzymes like peroxidase and laccase have an advantage over other enzymes or chemical processes because of their ability to completely mineralize various dyes to carbon-dioxide and water during degradation/decolorization process (Faraco *et al*., 2007; Krishnaveni *et al*., 2011, Ilić Đurđić *et al*., 2021).

Peroxidase is an oxidoreductase enzyme, the bound cofactor essential for its activity is haem. Peroxidase catalyzes the transfer of oxygen from the H_2O_2 to an appropriate substrate (like dyes) and consequently brings about oxidation of the substrate. Peroxidases can degrade/decolorize a wide range of industrial dyes because of their less-specific nature, as well as are able to transform or mineralize many other organo-pollutants (Lauber *et al*., 2017; Morsy *et al*., 2020). Hence, the aim of the present research work was to study the biodegradation/bio-decolorization of various industrial dyes using an extra-cellular peroxidase purified from a bacterial isolate *Bacillus* sp. F31 JX984444.1 which was isolated from onion waste and crude oil-polluted soil samples. Further, the purified bacterial peroxidase showed different specificities and efficiencies toward different industrial dyes.

Material and Methods

Organism

The bacterial isolate *Bacillus sp.* F31 JX984444.1 was originally isolated by serial dilution plate technique (Dhingra and Sinclair, 1993) using agar medium, from onion waste soil samples from District Mandi (Himachal Pradesh, India).

Preparation of crude enzyme

The seed culture of *Bacillus sp. F31 JX984444.1* was raised at 37°C (120 rpm) for 24 h. The 50 ml of production broth [containing beef extract (0.1%), glucose (1.4 %), yeast extract (0.2%), peptone (0.5%), NaCl (0.5), H_2O_2 (0.06%; v/v) with final pH of 7.5] was used to culture seed (10%, v/v). Then the culture seed was incubated for 2 days (48 h) under shaking conditions at 120 rpm at 37°C. Then the cell-free broth termed as crude peroxidase was extracted from harvesting incubated broth by centrifugation (10,000 X g for 20 min at 4°C; SIGMA 3K30, Germany).

Protein and peroxidase assay

Protein concentration was measured by a standard protein estimation method (Bradford, 1976) using Bovine serum albumin (BSA) 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 (Bao *et. al*., 2020). The A_{492} values were recorded and activity of the peroxidase was determined.

Enzyme unit of peroxidase

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 37ºC is known as one unit (U) of peroxidase enzyme.

Purification

The crude bacterial peroxidase (extracellular) produced from bacterial strain *Bacillus* sp. F31 JX984444.1 was purified by ammonium sulphate precipitation, dialysis and DEAE-cellulose column chromatography (anion-exchange chromatography). SDS-PAGE was used to determine molecular mass of purified peroxidase.

Application of an extracellular peroxidase (purified) from *Bacillus* sp. F31 JX984444.1 in degradation of dyes

Screening of industrial dyes for decolorization by purified peroxidase

To test the ability of purified peroxidase to decolorize selected industrial dyes, a total of 16 different 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), Methylene-Blue (MB), Black RL (BRL), Coomassie Brilliant Blue G-250 (CBBG), Direct Black-154 (DB) and Malachite Green (MG) were studied using dye decolorization assay mixture containing 50 µl of different dyes (20 µM), 700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2 and 0.75 U purified peroxidase to make the final volume 1 ml in different cuvettes. Decolorization of the test dye was assayed after incubation at 37ºC for 30 min, by observing the absorbance (A) at the respective wavelength (λmax) of the dye and decolorization (%) was determined as follows:

$$\textbf{\textit{Decolourization/Degradation}}\ \% = \frac{\textbf{\textit{Initial absorbance}} - \textbf{\textit{observed absorbance}}}{\textbf{\textit{Initial absorbance}}} \times \mathbf{100}$$

Reaction conditions optimization for dye degradation by purified peroxidase from *Bacillus sp. F31 JX984444.1*

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 various reaction conditions (or reaction parameters) such as temperature, reaction time, enzyme quantity, buffer system pH, effect of concertation of dye, effect of salt-ions on degradation of selected dyes and optimized conditions for dye degradation were ascertained.

Optimization of temperature (°C) for degradation of dye with purified peroxidase

To determine optimum temperature for degradation or decolorization of selected dyes (BF, RB, MB, CBBG and MG), the degradation reaction [1 ml reaction mixture containing 15 µl H_2O_2, 0.75 U purified peroxidase, 50 µl (20 µM) of different dyes and 700 µl (0.1 M) phosphate citrate buffer (pH 5.2)] was carried out at selected temperatures (25, 30, 35, 37, 40 and 45°C) in Eppendorf tubes in a

dry heating block for each dye, separately for 30 min. The decolorization was assayed after incubation of 30 min by measuring the absorbance at the respective wavelength of the dyes, and decolorization % was determined and analyzed.

Optimization of decolorization reaction time for degradation of dye with purified peroxidase

In order to determine the optimum reaction time for degradation or decolorization of few selected dyes (BF, RB and MB, CBBG, MG) the time of dye degradation assay [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 in 1 ml reaction mixture] 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.

Optimization of peroxidase (biocatalyst) concentration for degradation of dye with purified peroxidase

Purified peroxidase concentration was varied from 0.70 U to 1.1 U in 1 ml final reaction volume to perform the dye degradation assay [700 µl (0.1 M) phosphate citrate buffer (pH 5.2), 15 µl H_2O_2 and 50 µl (20 µM) of selected dye in 1 ml reaction mixture]. The decolorization (%) of different dyes was calculated after 35 min of incubation in dry bath 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.

Optimization of (buffer system or reaction buffer) pH for degradation of dye with purified peroxidase

Effect of reaction buffer (phosphate citrate buffer) pH on the dye decolouration of selected dyes by purified bacterial peroxidase (extracellular) 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.

Optimization of concentration (mg/ml) of selected dye for degradation of dye with purified peroxidase

The dye concentration of each selected dye was varied from 100 to 1000 mg/l [in dye decolouration assay mixture containing, 15 µl H_2O_2, 50 µl of different dyes, 700 µl (0.1 M) phosphate citrate buffer of optimised pH and optimized concentration purified peroxidase for each dye]. Optimized temperature, pH and time were used in decolorization assay for respective dyes and decolorization (%) was determined thereof.

Optimization of hydrogen peroxide concentration (mM) for degradation of dye with purified peroxidase

The concentration of H_2O_2 was varied from 0.25 mM to 3.50 mM in the decolorization reaction 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 (%) was determined in each case by using respective optimized parameters like temperature, pH and time for each of the dye in decolorization assay.

Effect of salt-ions and inhibitors for dye decolorization by purified peroxidase

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}, Mn^{+2}, Mg^{+2}, K^{+2}, Na^{+}, Hg^{+2}, Cu^{+2}, Fe^{+2}, Zn^{+2}, Ca^{+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 at 37°C in a dry heating bath for 30 minutes. Thereafter, the pre-incubated peroxidase was separately incubated with each of the selected dye in decolorization assay. Respective optimized temperature, pH and time for each of the dye were used in decolorization assay and decolorization (%) was determined in each case.

Results and Discussion

Purification

The purification of peroxidase from *Bacillus sp. F31 JX984444.1* 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 extracellular bacterial peroxidase was purified with a yield of 12.6 % up to 14.6-fold. The purified peroxidase was further used for dye decolorization experiments.

Application of an extracellular peroxidase (purified) from *Bacillus* sp. F31 JX984444.1 in degradation of industrial 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 37ºC 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). Therefore, it could be stated that the peroxidase of *Bacillus sp. F31 JX984444.1* has higher affinity for triphenyl methane dyes as substrates. It has been reported by the present studies that different types of peroxidase(s) decolorize specific type of dyes more efficiently as compared to others. The Triphenyl methane dyes were efficiently decolorized as compared to other groups of synthetic dyes by peroxidase from *Hevea brasiliensis* in a previous study (Chanwun *et al*., 2013; Blánquez *et al*., 2019). Many factors influence the decolourization of dyes by an enzyme 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 (Pereira *et al*., 2018; Ardila-leal *et al*., 2021). The dye 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.Many types of peroxidases purified from various microbial sources such as bacterial, fungi, yeast and plants etc. were found efficient to decolorize different industrial dyes (Fetyan *et al*., 2013; Telke *et al*., 2015, Pandey *et al*., 2016; Ilić Đurđić *et al*., 2021; Guo *et al*., 2021). The MnP (manganese-independent peroxidase) purified from *Dichomitus squalens* was also able to degrade selected azo dyes and anthraquinone dyes (Šušla *et al*., 2008). The MnP sourced from *Auricularia uricular-judae* was found to be highly stable and effective in degradation of very complex dyes (Liers *et al*., 2010). Peroxidase isolated from *Ganoderma cupreum* AG-1 (grown on decaying wood) was evaluated for its strong ability to degrade many azo dyes (Gahlout *et al*., 2013). A newly isolated and purified peroxidase from *Sterigmatomyces halophilus* (yeast strain), under the optimized conditions, showed a complete degradation efficiency on many azo dyes within 2 days (Al-Tohamy *et al*., 2021).

Reaction conditions optimization for dye degradation by purified peroxidase from *Bacillus sp. F31 JX984444.1*

Optimization of temperature (°C) for degradation of dye with purified peroxidase

Temperature of decolorization 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). The recorded data showed that purified peroxidase performed efficient decolourization of chosen common textile dyes at 30-40°C. The previous studies for dye degradation have proved that peroxidase works efficiently at a range of 25-45ºC for different dyes, though in some cases free peroxidase retained its activity at a temperature as low as 5°C (Carmen *et al*., 2012). 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 decolorization of different dyes by peroxidase from *Trametes versicolor* was found to be effective at 30°C (Celebi *et al*., 2013). The peroxidase from *Pleurotus ostreatus* (Shin *et al*., 1998) efficiently decolorized triphenyl methane dyes as BB and MB

Table 1: Screening of dyes for decolourization by purified peroxidase.

SN	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	*Dimethylanilin e*	-
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**

at 25ºC, whereas BF was 93% decolorized at 30ºC by peroxidase from *Aeromonas hydrophila* (Ogubue *et al*., 2012). The activity of different oxidative enzymes from different microbial sources such as manganese peroxidase, tyrosinase and laccase were evaluated for decolorization of Indanthrene Blue-RS and other industrial dyes, then the optimized temperature for decolorization reaction was found 37°C (Mohanty and Kumar, 2021). In general, peroxidase enzyme tends to lose its dye degradation capability beyond 45ºC and retained only about half of its activity at 65ºC (Yao *et. al*., 2013). This is possibly due to the reason that the higher temperature results in thermal inactivation of proteins (enzymes), which also affects cell structures and activity of enzymes (Shah *et al*., 2013).

Optimization of decolorization reaction time for degradation of dye with purified peroxidase

Reaction time of 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 (Fig. 2). The purified biocatalyst efficiently performed decolourization of selected dyes between 35-45 min at the chosen optimized temperature. The decolourization of the dye enhanced with the increase of reaction 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 effective and eco-friendly biodegradation of Reactive Black dye by peroxidase from *Sterigmatomyces halophilus* within effective rection time of 1-2 hours has been reported recently. It was also proved effective for textile azo dye wastewater processing and detoxifcation (Al-Tohamy *et al*., 2020). The chemical structure of dye was reported to have a relation with degradation time. Generally, dyes with low molecular weights and simple structures exhibited higher rates of dye removal than high molecular weight dyes (Chen *et al*., 2003).

Optimization of enzyme (biocatalyst) concentration for degradation of dye with purified peroxidase

The concentration of enzyme (purified peroxidase) 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 (Fig. 3). The removal of an organic pollutant (that act as a substrate for enzyme catalysis) is dependent on the amount of biocatalyst added and the contact time between enzyme and substrate. Increasing enzyme concentration will speed up the enzymatic reaction, as long as there is substrate available to bind. When all the substrate is bound, there will be no increase in the speed of reaction, since there will be nothing for additional enzymes to bind to. In a previous study, an increase in concentration of the Soyabean peroxidase in reaction mixture from 15 U/ml to 85 U/ml resulted in slow increase in the dye degradation (16-64%) that appeared to be constant at optimized dye concentration 80 U/ml (Kalsoom *et al*., 2013). Published studies showed that degradation of an

Table 2: Efficient degradation of selected dyes with purified peroxidase.

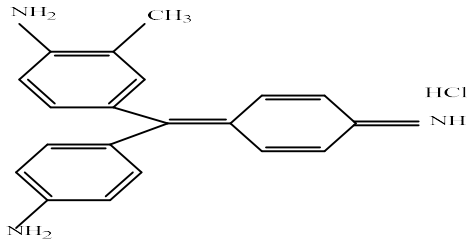
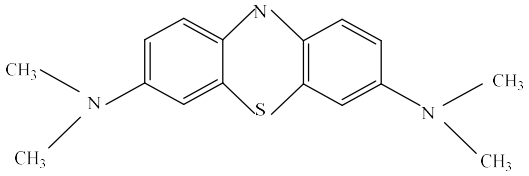
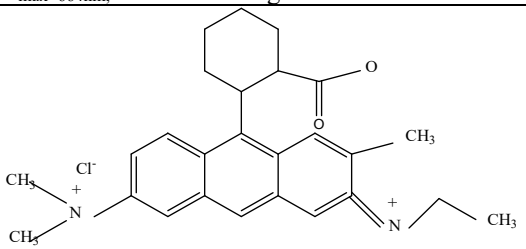
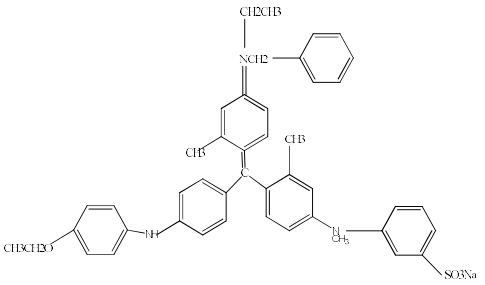
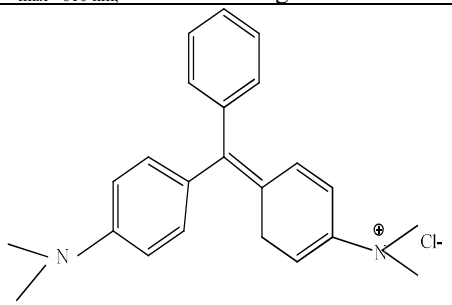
Dye	Structure of dye/ λ_{max}/ Molecular weight (g/mol)	Type of dye	Decolorization (%)
BF	 $\lambda_{max=545nm,}$ molecular weight= 337.85	Triphenyl methane dye	95.5
MB	 $\lambda_{max=664nm,}$ molecular weight= 319.85	Triphenyl methane dye	66.2
RB	 $\lambda_{max=\ 555nm,}$ molecular weight= 479.01	Polymeric/heterocyclic 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 salt-ions and inhibitors on degradation of dyes with purified peroxidase

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	80.7	84.1	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

azo dye required a much higher amount (15-times higher concentration) of enzyme than in the case of the anthraquinonic dyes (Mohan *et al*., 2005). Researchers found out that 3.3 µg/ml of peroxidase enzyme helped in the efficient removal of an anthraquinonic dye, the Reactive Blue 19 (Celebi *et al*., 2013).

Optimization of reaction buffer (phosphate citrate buffer) pH for degradation of dye with purified peroxiadse

The optimum pH of the assay buffer system for efficient decolorization of each selected dye (BF, RB, MB, CBBG and MG) with purified peroxidase was determined by varying buffer pH (phosphate citrate buffer) from 3-7. The optimum pH of reaction buffer for each of the selected dyes was 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) (Fig. 4). All the selected dyes were effectively decolorized in pH range 5.0-6.0 and at the higher values, the dyes showed less degradation. These results seem similar to what other researchers have reported (Marchis *et al*., 2011; Zhang *et al*., 2012; Tian *et al*., 2016) and suggested the usefulness of this enzyme to degrade industrial effluents or decolorize various industrial dyes which might be very acidic in nature. In another study, it was observed that strong to moderate acidic pH (pH 2-6) of buffer system is suitable for decolorization of most of the dyes including MG (Zucca *et al*., 2012). In the catalytic cycle of peroxidase all the reactions steps are pH dependent so enzyme was appeared to do decolorization best under acidic pH range (Kalsoom *et al*., 2013; Blánquez *et al*., 2019). In addition, HRP was reported to catalyze degradation of Remazol Blue at a pH of 2.5 (Šekuljica *et al*., 2020). The reaction steps of the catalytic cycle of peroxidase were pH-dependent and appeared to work best at an acidic pH. On checking pH, optimum researchers have obtained a bell-i shaped curve with an optimum at pH 5-5.5 (Kinnunen *et al*., 2017). Another study showed the lignin-peroxidase to work at an optimum pH of 3, where it was used to degrade azure B dye (Silva *et al*., 2013).

Each enzyme has an optimum pH range. Changing the pH beyond of this range will slow enzyme activity. Extreme pH values can cause denaturation of enzymes. At native state an enzyme is a combination of both cationic and anionic groups at any given pH. These groups are part of the active site of an enzyme (Gomare *et al*. 2008). The rate of enzymatic reaction varies with the changes in the pH of the reaction medium because the ionic state of active site in enzyme changes hence, activity of enzyme is also changed (Hossain and

Anantharaman. 2006). Moreover, these organic dyes have different redox potentials, which may also affect the decolorization activity of the designed heme enzyme (Jenkins *et al*., 2021).

Optimization of selected dye concentration (mg/ml) for degradation of dye with purified peroxidase

In the present work, the maximum decolorization was found in case of BF (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). In the present work, the maximum decolorization was found in case of dye BF (97.1%) The results were in accordance with a previous study, which showed that dye-degradation efficiency of enzyme peroxidase decreased with increasing concentration of dye, then eventually resulted in an inhibition effect, thus presenting less degradation (Boucherit *et al.*, 2013). Other microbial peroxidase showed similar results where the degradation rate decreased with an increase in dye concentration (Silva *et al*., 2012). Enzyme-catalysed reactions were affected by the concentration of the substrate present in the aqueous phase (Robinson *et al*., 2015). It is appeared quite possible due to the reason that if the substrate concentration is slowly increased and the concentration of enzyme is kept constant the reaction rate would continue to increase until it reaches equilibrium state. At equilibrium, there will be maximum rate of reaction after that any further addition of the substrate would not increase the rate of reaction. The effect of dye concentration in reaction mixture is an important consideration for its application in industrial dye removal or decolorization. In effluent from textile printing house, the dye concentration is approximately 100-800 mg/l (Zhao and Hardin, 2007). According to a study, with increase in dye concentration, the decolourization efficiency of dye decreased 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 of various industrial dyes was due to breakdown of dyes into unknown colourless products (Fetyan *et al*., 2013).

Optimization of hydrogen peroxide concentration (mM) for degradation of dye with purified peroxidase

H_2O_2 reacts with the native enzyme (purified peroxidase from *Bacillus* sp. F31 JX984444.1) to form an enzyme intermediate, which easily combine with an aromatic compound such as dyes to carry out its oxidation and produce a free radical form. To figure out an appropriate concentration of H_2O_2 is definitely a key factor in obtaining a higher rate of dye degradation (Yao *et al*., 2013). In this regard, in present study experiments were done to decolouration/ degradation of the selected textile dyes (BF, RB, MB, CBBG and MG) the H_2O_2 concentration varied while keeping the other reaction conditions of degradation reaction constant. The concentration of H_2O_2 was varied from 0.25 mM 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 (Fig. 6). Activity of Peroxidase is known to change considerably with change in the concentration of the presence of H_2O_2 (Gholami-Borujeni *et al*., 2011). Many harmful organic compounds including industrial dyes can be oxidised and degrade/ decolourize by using H_2O_2 alone or in conjunction with other materials. The addition of a small amount H_2O_2 in the reaction mixture may lead to the generation of free radicals like •OH that facilitates faster degradation of many organic compounds. On the other hand, higher concentration of H_2O_2 was detrimental to the reaction process, most likely due to the damage to the enzyme being a protein itself. So, it becomes necessary 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*., 2012). In case of Soya bean peroxidase, increase in H_2O_2 concentration in

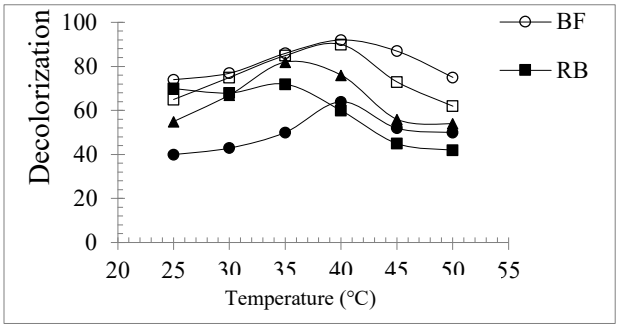


Figure 1: Optimization of temperature (°C) for dgradtion of dye with purified peroxidase.

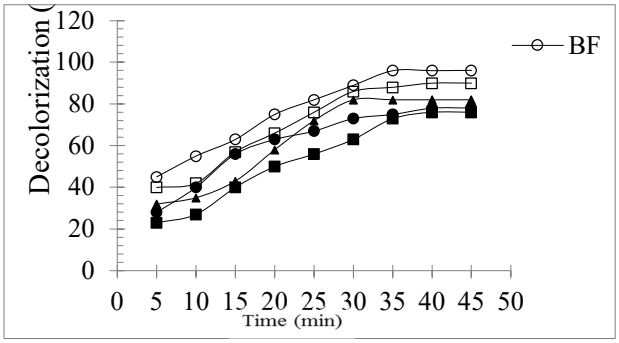


Figure 2: Optimization of reaction time for degradation of dye with purified peroxidase.

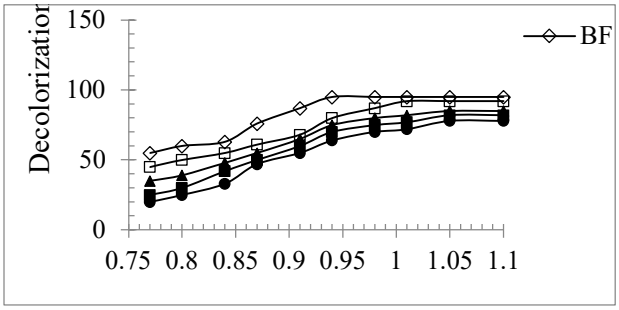


Figure 3: Optimization of biocatalyst (Enzyme) concentration for degradation of dye with purified peroxidase.

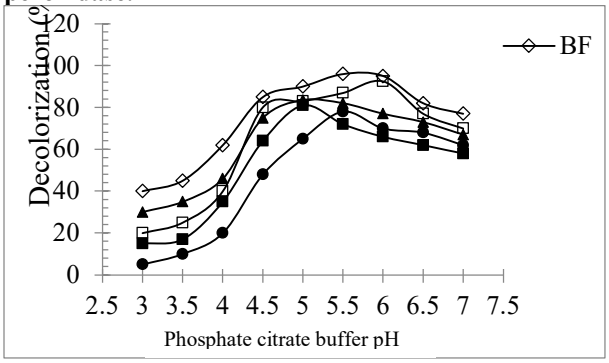


Figure 4: Optimization of reaction buffer (phosphate citrate buffer) pH for degradation of dye with purified peroxidase.

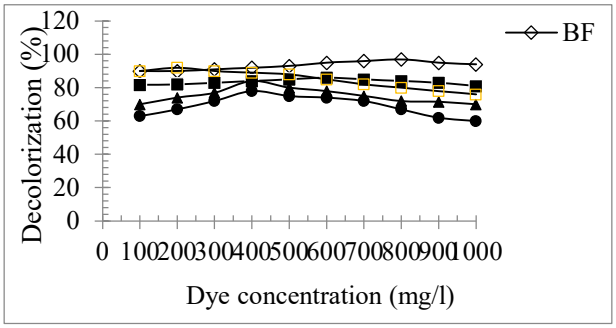


Figure 5: Optimization of dye concentration (mg/ml) for degradation of dye with purified peroxidase.

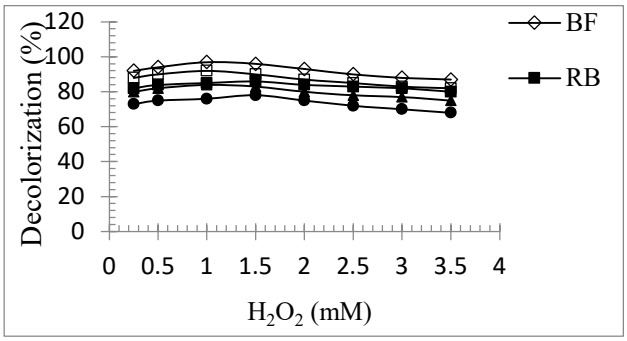


Figure 6: Optimization of hydrogen peroxide concentration (mM) for dye degradation with purified peroxidase.

reaction mixture led to an increased dye decolourization. At 64µM concentration of H_2O_2 maximum dye decolourization was observed, any further increase in H_2O_2 concentration did not cause any additional effect (increase /decrease) on dye decolorization (Kalsoom *et al*., 2012).

Effect of salt-ions and inhibitors on dye decolorization by purified peroxidase

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 decolorization of all the five dyes with 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%), (Table 3). As in previous study, the decolourization was slightly stimulated for extracellular purified peroxidase in the presence of some bivalent metal ions such as

Mg^{+2}, Zn^{+2} and Co^{+2}, 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, decolorization of MG by peroxidase from *Pseudomonas* sp. was observed to significantly enhanced in the presence of Mg^{+2} and Mn^{+2} (1-2 mM) ions (Du *et al*., 2011).

Different industries like textile, food, pharmaceuticals, cosmetics etc., release a huge amount of the effluents daily in the form of wastewater into rivers and other water reservoirs causing serious health issues. That leads to enhanced biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of water and affect the ecosystem drastically. Therefore, it is very difficult to treat textile industry effluents because of their high BOD, COD, heat, color, pH and the presence of metal ions (Anjali *et al*., 2007). Treatment of various industrial effluents discharged with harmful compounds like synthetic dyes becomes necessary before their final discharge into the environment and different water sources. There are many conventional methods for the effective removal of dyes such as degradation of dye by anaerobic reaction, adsorption of dye by activated carbon, silica, functional polymer granules, biomaterials, nanofiltration, oxidation and precipitation by Fenton's reagent, bleaching with chloride, ozone photo degradation and membrane filtration (Robinson *et al*., 2001). These methods have been outdated because of many drawbacks such as low efficiency, high cost and in-applicability to a wide variety of complex hazardous dyes. In recent years a number of studies have focused on some microorganisms capable of degrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolonization of dyes (Shah *et al*., 2013; Šekuljica *et al*., 2020: Chauhan & Kanwar 2020; Barathi *et al*., 2020). Many types of peroxidases purified from various sources were reported to decolorize different industrial dyes (Shin *et al*., 1998; Hong *et al*., 2012; Celebi *et al.,* 2013, Salvachúa *et al*., 2013; Chen & Li, 2016). The MnP purified from *Dichomitus squalens* was also able to degrade selected azo and anthraquinone dyes (Šušla *et al*., 2008). A thermostable peroxidase from *Bacillus stearothermophilus* and peroxidase sourced from *Auricularia auricula-judae* has been found to be stable in decolourization of the many complex structured industrial dyes such as Reactive Black-5 and Reactive Blue-5 (Loprasert *et al*., 1988; Liers *et al*., 2010). Peroxidase isolated from *Ganoderma cupreum* AG-1 was evaluated for its ability to decolorize many industrial azo dyes Gahlout *et al*., (2013). In some another previous studies, bacterial and fungal peroxidase(s) have been found to be very efficient biological decolourization tools (Zucca *et al*., 2012; Saladino *et al*., 2013, Lauber *et al*., 2017, Morsy *et al*., 2020). The decolorization or degradation of industrial dyes by using biocatalysts or enzymes from various sources like bacteria, fungi, yeast and even plants is an eco-friendly and low-cost process. Biocatalytic methods of decolorization also produce a low quantity of by-products as compare to physical, chemical and photochemical approaches.

Present results suggested that the extracellular peroxidase (purified) from *Bacillus* sp. F31 JX984444.1 was effective in discolouration of many common textile dyes. The purified bacterial peroxidase assisted dye-degradation parameter(s) optimization resulted in increased dye degradation. The optimized dye-degradation process was most successful for BF, where 97.1% of dye decolorization was achieved and minimum decolorization 78.1% was found in case of MG. The selected dyes for decolorization experiments i.e., BF, RB and MG are basic triphenyl methane dyes mostly used in textile, pharmaceutical and chemical industries; while RB is a cationic triphenylmethane dye used in textile industries for dying, wool, silk, nylon 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. The great potential of peroxidase produced by *Bacillus* sp. F31 could offer a cheaper and efficient alternative treatment of wastewaters contaminated heavily with industrial dyes. Further extension of work shall comprise the study of the combination of the extracted enzyme(s) with other established methods for wastewater management to see if enzyme alone or in combination with other methods m ay be helpful as an industrially efficient treatment procedure for efficient dye-degradation.

Conclusion

In present study the purified peroxidase from Bacillus sp. F31 was found to be highly effective in decolourisation/degradation of 5 different industrial dyes (BF, RB, CBBG, MG and MB). This approach will provide an effective application of peroxidase in managing industrial effluents containing dyes. Proper management of industrial effluents by using peroxidase can give environmental and economic benefits.

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Conflict of interest

The authors declare that they have no conflict of interest.

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