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Original Research

Bioremediation of Congo red using Polyvinyl alcohol - Chitosan Supported Peroxidase as an Efficient and Reusable Catalyst

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Abstract: The discharge of textile effluents containing azo dyes is critical due to their persistence in wastewater and carcinogenic, mutagenic impacts on aquatic organisms. Considering the toxicity of azo dye, an effective remediation strategy should be applied before disposing into the environment. Among all the advance techniques like electrochemical degradation, fenton oxidation, photocatalysis, ozonation etc., biodegradation using biocatalyst found to be an eco-friendly and economic process to deal with this problem. Along with advantages, biocatalysts particularly enzymes face limitations like instability, single-use restriction, and reduced efficiency under operational conditions. Immobilization addresses these challenges by enhancing enzyme stability, reusability, and catalytic performance. The present study focuses on the development of an efficient bioremediation approach for the removal of Congo red dye from aqueous solutions using peroxidase (HGP) extracted from germinated Macrotyloma uniflorum (horse gram) seedlings. The enzyme was immobilized on polyvinyl alcohol–chitosan beads through epichlorohydrin-mediated crosslinking, enabling its application as a reusable biocatalyst for dye degradation. The immobilization method achieved high efficiency with 96% enzyme retention. The immobilized peroxidase exhibited enhanced stability and was evaluated for its efficacy in degrading Congo red dye. Under optimized conditions, 26 units of immobilized peroxidase achieved complete decolorization (100%) of a 160 mg/L Congo red solution within 10 minutes at 28°C and pH 4. Environmental safety of the degradation products was confirmed through phytotoxicity and microbial growth assessments. Additionally, the immobilized enzyme retained its catalytic activity across eight successive cycles, underscoring its reusability and potential for practical applications in bioremediation of dye-contaminated wastewater. A newly developed biocatalyst demonstrates simple method of preparation, environmental benignity, biocompatibility, high efficiency, enhanced stability, and facile recyclability.

Key Words	Horse gram peroxidase, polyvinyl alcohol-chitosan immobilization, Epichlorohydrin cross-linking, Congo red dye degradation, wastewater bioremediation, enhanced reusability
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1. INTRODUCTION

Contamination of soil, water, and air by industrial effluents from various chemical industries is one of the major concerns of the 21st century. Among various chemical industries, azo dye industries are one of the major contributors to environmental pollution(Carliell et al. 1995). Azo dyes are extensively employed in both the textile and food industries due to their appealing vibrant shades and impressive colorfastness properties (Chen, 2006). However, their widespread use comes with a significant environmental challenge. Azo dyes possess high water solubility and resist easy biodegradation, leading to the generation of wastewater during their production and industrial application. This wastewater, if released directly into water bodies, poses a grave threat to aquatic ecosystems and human health (Kaushik & Malik, 2009; Somasekhara Reddy, Sivaramakrishna & Varada Reddy, 2012; Chatha, Asgher & Iqbal, 2017). It is imperative to address this issue, as the pollution caused by these non-biodegradable dyes can have harmful effects on both our environment and well-being. It is well known that, it is responsible for many health conditions like skin, eye and gastrointestinal irritations, blood clotting, mutation, reproduction related diseases, respiratory problems (Mittal et al. 2009; Li, Chen & Wang, 2010) etc. Considering the serious environmental and health impacts associated with the effluents from azo dyes industries, development of protocols for the total removal of azo dyes from the effluents is highly desirable (Hai, Yamamoto & Fukushi, 2006; Rajkumar & Kim, 2006).

Effective strategies for remediation of dye-contaminated waste water are use of oxidizing agents, adsorption of pollutants over various supports and photocatalytic treatments (Chanathaworn et al. 2012; Nidheesh, Gandhimathi & Ramesh, 2013; Fisli et al. 2014; Ojedokun & Bello, 2017; Lubbad, Al-Roos & Kodeh, 2019; Cao et al. 2024, Haroon et al. 2023, Jadon et al. 2022) etc. These conventional procedures suffer from the drawbacks like creation of sludge in significant amount, high cost, cumbersome operational procedures, toxic end products (Ahmedi et al. 2015) etc. In recent years, in this context ecofriendly biological methods involving the use of enzymes for cleaning up pollutants has gained considerable interest (Bhandari et al. 2021). This is because, enzymatic treatments for bioremediation are known to offer advantages like mild reaction conditions, substrate specificity, cost effectiveness (Esposito & Durán, 2000; Husain & Jan, 2000) etc. At the same time, poor stability of enzymes at higher temperature, higher cost of their isolation and difficulties in their recovery from the reaction media limit their use on industrial scale (Chiong et al. 2016, Long, H., Li, X., Liu, X., Wang, W., Yang, X., & Wu, 2023). As enzyme stability and its recovery for possible reuse can be enhanced enormously by immobilizing the enzyme on suitable solid support, development of suitably tailored immobilized enzymes for bioremediation pollutants has become an interesting area of research.

Peroxidase, an oxido-reductase class of enzyme, is abundantly found in plants like horseradish, white radish, bitter gourd, garlic, tomato, soya bean, turnip (Husain, 2010; Osuji et al. 2014) etc. Owing to their wide substrate specificity and excellent bio-catalytic activities, plant peroxidases are the molecules of commercial interest (Akhtar, Khan & Husain, 2005, Ali et al. 2018, Zhang et al. 2010). Especially, the ability of peroxidases to catalyze oxidation at low concentrations make them enzyme of choice for bioremediation of variety of synthetic dyes (Ram Singh, Taranjeet Singh, 2019). In light of these considerations, this study aimed to develop a potential bioremediation process for degradation of Congo red dye using solid-supported peroxidase enzyme.

In the present study, peroxidase enzyme (HGP) was extracted from Macrotyloma uniflorum (Horse Gram) and was crosslinked on PVA-Chitosan beads using epichlorohydrin as a crosslinker. The potential of crosslinked peroxidase enzyme was investigated in degradation of model dye Congo red. A simple degradation process of Congo red dye without use of any mediator (TEMPO, HOBt), was developed using immobilized peroxidase enzyme and hydrogen peroxide.

2. MATERIALS AND METHODS

Congo red ($C_{32}H_{22}N_6Na_2O_6S_2$), hydrogen peroxide 30% w/v (H_2O_2), ammonium sulphate AR ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), o-Phenylenediamine, glacial acetic acid AR (CH3COOH), Polyvinyl alcohol

(Molecular weight approx. 14,000), epichlorohydrin (C3H5ClO) were supplied by MOLYCHEM (Mumbai, India), chitosan ((C6H11NO4)n) from shrimp shells was purchased from Himedia,, horse gram (Macrotyloma uniflorum) was purchased from local market and allowed to germinate for minimum 2 days.

For all the experiments, different concentrations of dye solutions ranging from 20 ppm to 200 ppm were prepared with deionized water and these solutions were stored protected from light at 4 °C but no special treatment was given to dye solutions. Extraction of HGP from horse gram was performed using ammonium sulfate precipitation method. Dye degradation study was evaluated using UV-Visible spectrophotometer and HPLC.

2.1 Extraction and purification of Peroxidase enzyme from Horse gram (HGP)

For isolation of peroxidase enzyme, germinating horse gram (Macrotyloma uniflorum) seedlings were used. Seeds were soaked on cotton bed in saline solution for 48 hours. Crude extract was prepared by homogenization using buffer saline solution (Phosphate buffer saline pH 7.4, 0.2M). Crude extract was treated with ammonium sulfate for 30% saturation. After centrifugation, supernatant subjected to 80% saturation. Precipitate was collected after centrifugation and dialyzed and partially purified enzyme used for further optimization.

2.2 Preparation of Polyvinyl (PVA)-Chitosan beads as support

Chitosan beads were selected as a support due to their biocompatibility and multiple active binding sites. However, the formed chitosan beads exhibited relative fragility. To enhance their structural stability throughout the process, polyvinyl alcohol (PVA) was added. Chitosan and PVA solutions were prepared by dissolving each in glacial acetic acid and distilled water respectively. These two homogenous solutions were mixed in 1:1 proportion followed by stirring for 1 hour at room temperature. The obtained homogenous mixture was then added dropwise through a syringe into 1N NaOH solution to get PVA-chitosan beads. These beads were allowed to stand in same solution for 2 to 3 hours for hardening. The hardened beads were then washed with distilled water till neutral pH.

2.3 Immobilization of extracted peroxidase enzyme on PVA-Chitosan beads

Immobilization of extracted peroxidase enzyme was performed by crosslinking method. PVA-chitosan beads were then treated with 2% Epichlorohydrin solution for 2 hours with occasional stirring. After treatment beads were washed with distilled water twice and suspended in 5 ml phosphate buffer of pH 7.4 containing 0.2 ml (26U) peroxidase enzyme. To ensure immobilization, this system was kept overnight at cold condition. Beads were then washed twice with 5 ml of distilled water to remove unbound enzyme and stored in phosphate buffer (pH 7.4) at 4°C till further use. The washings were collected to measure residual peroxidase activity. Percent immobilization was then calculated by subtracting the activity lost in the washings from the initial enzyme activity used for immobilization. For comparative evaluation of immobilization techniques, peroxidase from horse gram seedlings (HGP) was also immobilized on sodium alginate beads via the entrapment method.

2.4 Assay of free HGP and immobilized peroxidase enzyme

Peroxidase activity and stability of crosslinked HGP were determined and compared to that of the free enzyme and HGP immobilized on sodium alginate by entrapment method.

Study of activity of free HGP and HGP immobilized on PVA-Chitosan support and sodium alginate support was performed using o-Phenylenediamine (OPD) as a substrate at room temperature ((Gutema Dinkisa Idesa, 2018)

The procedure followed was as outlined below. The assay mixture comprised 0.4 mg/mL o-phenylenediamine (OPD), 0.1 mL of 30% hydrogen peroxide, 0.9 mL of distilled water, and 0.2 mL (26 U) of free enzyme. After dilution with an additional 1.0 mL of distilled water, the absorption was measured at 492 nm following a 1-minute incubation.

The same procedure was performed for assay of immobilized enzyme where 0.2 ml of free enzyme immobilized on sodium alginate beads and PVA-chitosan beads were used for assay. Activity of free and immobilized enzyme was checked for 7 days to study the stability of enzyme.

2.5 Aqueous phase degradation of Congo red dye using HGP

The dye degradation reaction was carried out in a 100 ml beaker containing 10 ml of dye solution, buffer solution and fixed amount of enzyme. To this, 0.3% H_2O_2 was added dropwise, and reaction mixture was stirred continuously. 20 µl of sample were taken at regular interval and the dye degradation reaction was monitored using UV–visible spectrophotometer (Shimadzu UV-1800 Japan) at 570 nm.

The rection parameters like pH (2–10), Congo red dye concentration (20–200 mg/L), H_2O_2 dose (0.0 ml–1.0ml), dose of free enzyme (0.1 ml-1.0 ml), and reaction time (10 to 90 minutes) were studied. The percentage degradation was calculated using formula,

$$\text{Dye removal (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 and A_t are the absorbance before and after enzymatic treatment, respectively.

The degraded dye was characterized by HPLC technique and compared with HPLC spectra of Congo red.

2.6 Toxicity study

Phytotoxicity study of Congo red was performed before and after enzymatic treatment using vigna aconitifolia seeds. For this, 10 seed of vigna aconitifolia were allowed to germinate in Congo red dye solution and supernatant obtained after dye degradation. Both samples were compared with standard sample germinated in distilled water.

For microbial growth study of Congo red, two microorganisms were selected to check growth, S.aureus and E.coli. The inoculum was allowed to grow on nutrient agar using standard plating technique and growth was compared with dye solution and supernatant obtained after dye degradation.

3. RESULTS OR RESULTS AND DISCUSSIONS

This section can be organized using subheadings. It should present a clear and concise description of the experimental results, their interpretation, and the conclusions derived from them. If the flow of the paper suggests to combine the results and discussions in one Section you can do it here.

3.1. Extraction and immobilization of HGP

3.1.1. Extraction of HGP (Protein estimation)

Protein content estimation of extracted HGP was performed at each step of extraction by Folin Lowry method (LOWRY *et al.*1951). Enzyme activity was quantified by measuring the change in optical density (OD) over time, with activity calculated using the Beer-Lambert law to determine concentration changes. One enzyme unit was defined as the amount catalyzing the transformation of 1 µmol of substrate per minute un-der the assay conditions. Highest protein content was observed in case of dialyzed enzyme than crude extract. Enzyme obtained after dialysis showed activity of 130 units/ml.

3.1.2 HGP immobilization study

Immobilization of peroxidase is essential to enhance its operational stability, reusability, and resistance to environmental fluctuations. This technique not only extends the enzyme's functional lifespan but also facilitates its repeated use, which is crucial for efficient and cost-effective bioremediation processes. With this objective the immobilization of extracted HGP was studied by two methods. Immobilization of HGP on sodium alginate

beads by entrapment method and immobilization of HGP on PVA-Chitosan beads by crosslinking method. The results were compared with the assay of free HGP.

It was observed that the synthesized PVA-chitosan beads are effective in immobilization of peroxidase enzyme and gave 96% (table 1, entry 2) immobilization whereas the immobilization of peroxidase enzyme by entrapment method on sodium alginate 81% (table 1, entry 3) immobilization. Thus, comparison of two methods showed that immobilization of peroxidase enzyme by crosslinking method has advantage of better percent immobilization (96%) compared to immobilization of peroxidase enzyme by entrapment method (80%). The table shows the comparative study of assay of free HGP, HGP immobilized on PVA-Chitosan beads and HGP immobilized on sodium alginate beads.

Table 1: Comparative % immobilization of peroxidase enzyme immobilized on solid support.

Entry	Enzyme	Free enzyme concentration (in mL)	Free enzyme concentration (Units)	Enzyme concentration after immobilization (Units)	% immobilization
1	Free HGP	0.2	26	-	-
2	HGP immobilized on PVA-Chitosan beads	0.2	26	25	96%
3	HGP immobilized on sodium alginate beads	0.2	26	21	81%

3.2 Comparison of activity and stability of free and immobilized HGP

The prime aim of enzyme immobilization is to enhance stability, yet it can potentially lead to a reduction in enzyme activity. Both enzyme activity and stability hold vital significance, and a robust immobilization technique helps in achieving stability while maintaining activity levels that are comparable. HGP was immobilized using two immobilization techniques, crosslinking method and entrapment method. To check the efficacy of immobilization technique used, the activity and stability of crosslinked HGP and entrapped HGP was checked and compared with free HGP by performing an assay using OPD. An assay was performed for 7 days with 24 hours interval between two screenings. The comparative results of enzyme assay of free HGP, crosslinked HGP and entrapped HGP are shown in Fig.1. The results indicated that the activity of crosslinked HGP is comparable to that of free enzyme and showed improved stability.

For free enzymes, plot of absorbance vs days showed exponential decrease in activity of HGP with time. After 5 days free HGP loses more than half of its original activity. In comparison to free HGP, the activity of crosslinked enzyme remained consistent for 72 hours and it remained stable for almost 7 days **[Fig.1]**. While in case of entrapped enzyme, activity start decreasing after 24 hours. This indicates that the immobilization technique used is suitable for immobilizing peroxidase and improves stability of enzyme.

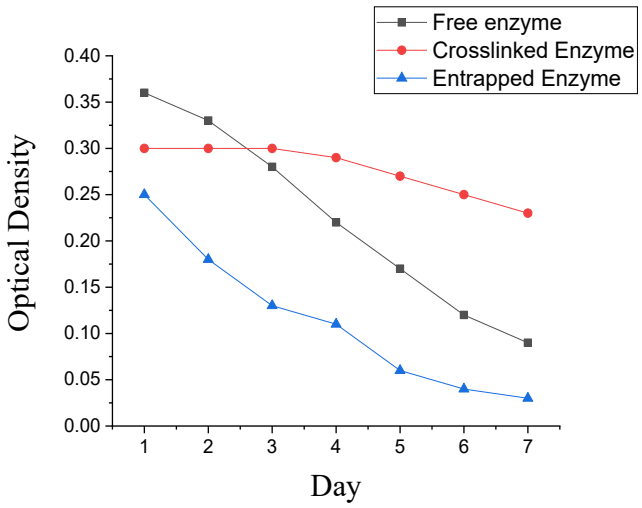


Fig. 1: Activity comparison between free HGP, HGP immobilized using crosslinking method and HGP immobilized using entrapment method

3.3 Aqueous phase degradation of Congo red dye using free HGP

To evaluate the efficacy of the enzyme in degrading Congo red dye, preliminary experiments were conducted using the extracted free enzyme. Key parameters, including contact time, pH, and concentra-tions of dye, enzyme, and H_2O_2, were optimized. Once these conditions were established, the performance of cross-linked HGP immobilized on PVA-chitosan beads was assessed under the optimized conditions.

3.3.1 Optimum contact time

The optimal contact time required for the dye removal was evaluated initially. To a sample solution bearing 10 ml of 40 ppm dye solution, 0.2 ml of enzyme and buffer solution of pH 4, 1 ml 0.3% H2O2 was added dropwise and allowed to stand for 10 to 90 minutes. After each interval of 10 minutes, a small amount of sample solution was taken and examined for the residual dye concentration. Maximum dye degradation was observed within the initial 10-minute interval, with no significant change in degradation observed up to 90 minutes. Consequently, for subsequent optimization studies, the contact time was set to 10 minutes.

3.3.2 Optimum pH

Enzymes exhibit optimal activity within a specific pH range. To fix the optimal pH range of extracted HGP for Congo red dye degradation, pH optimization study was carried out on aqueous solution of Congo red dye. For this series of buffers ranging from pH 2 to pH 10 were prepared and fixed amount of each buffer was added in 9 different vials containing 10 ml of 40 ppm Congo red dye and 0.2 ml of free enzyme. To each of the nine vials, 1.0 ml of 0.3% H_2O_2 was added gradually with constant stirring and percentage degradation at various pH were studied. **[Fig.2]** illustrates the variation in dye degradation across different pH values. The results indicate that approximately 100% dye removal occurred at pH 4 due to the HGP-catalyzed reaction in decreased at pH levels below 4 and above 5. Since maximum degradation was achieved at pH 4, a buffer of pH 4 was selected for subsequent experiments.

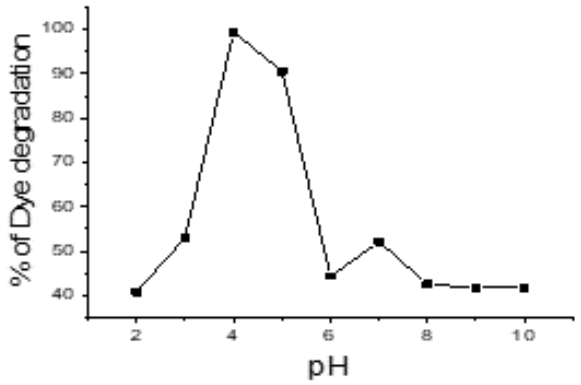


Fig. 2: Effect of pH on dye degradation using HGP

3.3.3 Optimization of H_2O_2 dose

Preliminary studies on Congo red dye degradation indicated that 0.3% H_2O_2 is sufficient to achieve effective degradation. Employing such mild conditions is advantageous, particularly in minimizing environmental impact. To find out the minimum amount of H_2O_2 required for dye degradation, optimization of amount of H_2O_2 dose was carried out by keeping all the other experimental conditions constant. Thus, a series of doses ranging from 0.0 ml to 1.0 ml were gradually added in 6 different vials containing 10 ml of 160 ppm Congo red dye solution, 0.2 ml (26U) of enzyme and 4 ml buffer of optimum pH**. [Fig.3]** represents graph of dye degradation as function of H_2O_2 dose. 0.2 ml of H_2O_2 was found to be adequate for the maximal dye degradation at the experimental conditions defined before.

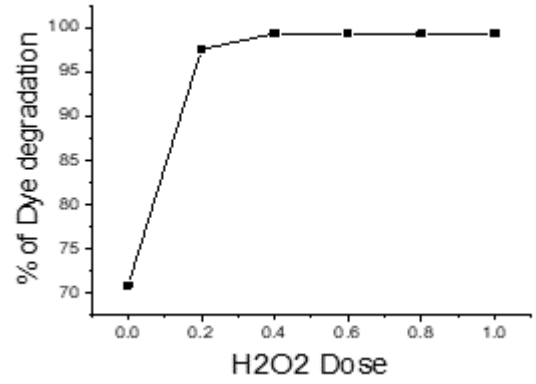


Fig. 3: Effect of H_2O_2 on dye degradation

3.3.5 Optimization of free enzyme dose

The amount of catalyst utilized for dye degradation holds significant importance. Considering the high cost of enzyme, it is preferable for the enzyme to facilitate dye degradation at lower concentrations. Optimization of enzyme dose was done by varying enzyme concentration. A series of doses of 0.1 ml (13U), 0.2 ml (26U), 0.4 ml (52U), 0.6 ml (78U), 0.8 ml (104U) and 1.0 ml (130U) of HGP were gradually added in 6 different vials containing 10ml of 160 ppm Congo red dye solution 0.2 ml of 0.3% H2O2 and 4ml buffer of optimum pH. [Fig.4] represents percentage dye degradation as function of concentration of enzyme. It was observed that 0.2 ml i.e 26 units of enzyme is sufficient to bring about 100% dye degradation.

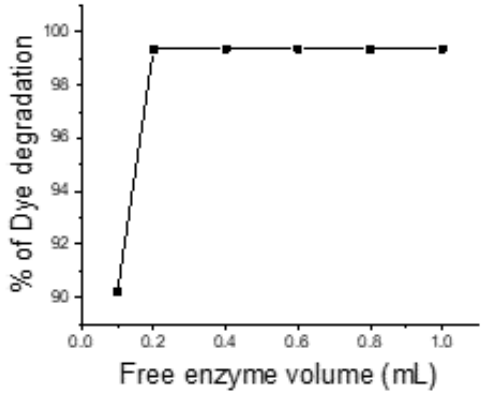


Fig. 4: Concentration of enzyme required for dye degradation

3.3.6 Optimization of Congo red dye concentration

To determine the maximum concentration of Congo red dye that can be degraded by free HGP enzyme, a series of Congo red dye solutions were prepared with concentrations ranging from 20 to 200 ppm. The enzyme concentration and H2O2 were maintained at constant values. For each concentration, a 10 ml aqueous solution of Congo red dye was combined with 4.0 ml of pH 4 buffer, 0.2 ml of free enzyme, and 1.0 ml of H2O2. This mixture was stirred for 10 minutes, and the dye degradation was monitored. This study revealed that complete removal of the dye was achieved up to a concentration of 160 ppm [Fig.5].

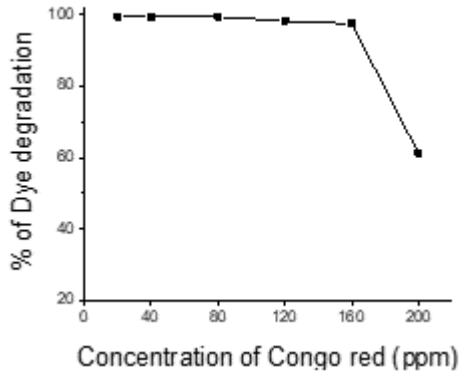


Fig. 5: Effect of dye concentrations on dye degradation using HGP

3.4 Congo red degradation using immobilized HGP

The efficacy of immobilized HGP for Congo red degradation was studied at optimized condition and it was observed that immobilized HGP showed similar results as shown by free HGP. For 100% removal of dye using immobilized enzyme, contact time was found to be 10 mins.

3.4.1 Reusability Study

Achieving economic viability in the process remains a central goal in any enzyme catalyzed reactions. A proficient immobilization technique contributes to enhancing the enzyme's stability and its capacity for multiple uses. In the present study, it was observed that immobilized enzyme can be successfully reused for 8 cycles achieving almost 100% removal for first six runs, 98% and 85% for 7th and 8th run respectively **[Fig.6].**

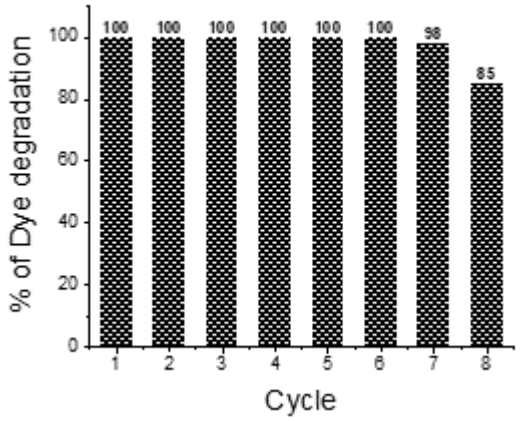


Fig. 6: Reusability study of immobilized peroxidase enzyme

3.5 Dye degradation study

3.5.1 Dye degradation study

UV-Visible spectrum of control and sample were observed at 570 nm as Congo red has maximum absorption at 570 nm in acid medium. The absence of absorption peak at 570 nm indicates almost complete removal of Congo red from reaction mixture [Fig.7].

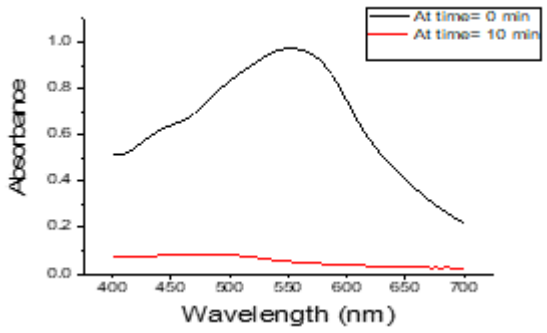
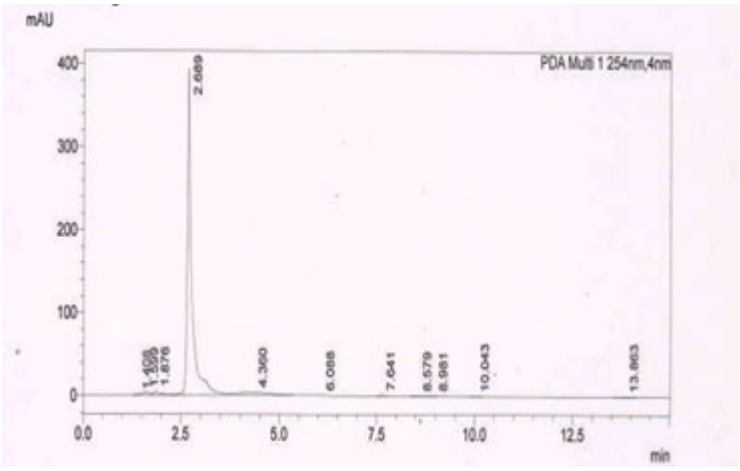


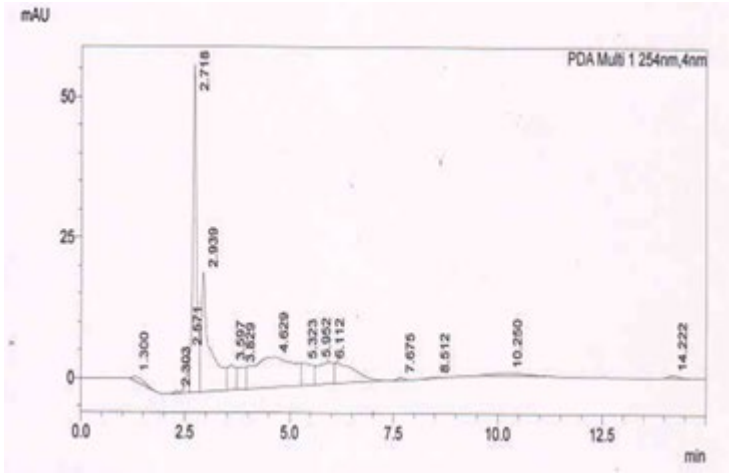
Fig. 7: UV-Visible spectrum of Congo red and reaction mixture

3.5.2 HPLC analysis

Being a part of oxidoreductase class of enzymes, HGP follows oxidative pathway for degradation of Congo red(Ahmedi et al. 2012). HPLC analysis was performed to confirm Congo red degradation. Chromatogram of control i.e. Congo red dye and dye solution after HGP treatment was recorded at retention time 2.5 min. **[Fig.8]**. The reduction in peak height that is reduction in absorption indicates decrease in concentration of model dye. Appearance of extra peaks in chromatogram of HGP treated solution indicates degradation of parent molecule.



(a)



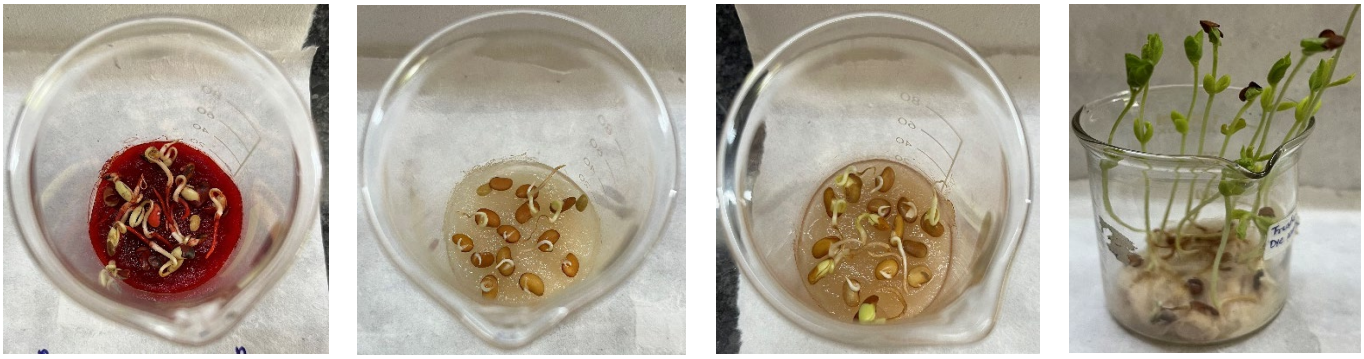
(b)

Fig. 8: HPLC Chromatogram of (a) Congo Red dye (b) degraded dye

3.5.3 Toxicity study

For any enzymatic dye degradation process, it is necessary to check the safety of treated dye solution before discharging it into natural water sources. Therefore, the supernatant solution after enzymatic degradation of Congo red was tested for phytotoxicity and microbial growth study. Phytotoxicity study of Congo red dye solution was performed before and after enzymatic treatment using Macrotyloma uniflorum seeds.

The obtained results indicate that Congo red badly affect the seedlings growth by penetrating cells **[Fig.9a].** While Macrotyloma uniflorum seeds grown in supernatant obtained after dye degradation showed comparable growth as per distilled water **[Fig.9(b & c)]**. The average root length obtained ranges between 2 cm to 3 cm ± 2 mm. In a similar way, microbial study gave favorable outcomes for supernatant obtained after dye degradation. The zone of inhibition for the dye solution before treatment was measured at 3 ± 0.5 mm. However, no microbial growth inhibition was observed after treatment. Study showed that Congo red dye solution inhibit growth of microorganism whereas supernatant obtained after enzymatic treatment showed proper growth of microorganisms **[Fig.10]** indicating that it is nontoxic.



(a) (b) (c) (d)

Fig. 9: Phytotoxicity study of (a) Dye solution (b) Supernatant obtained after enzymatic degradation (c) water (d) plant growth in Supernatant obtained after enzymatic degradation after 10 days

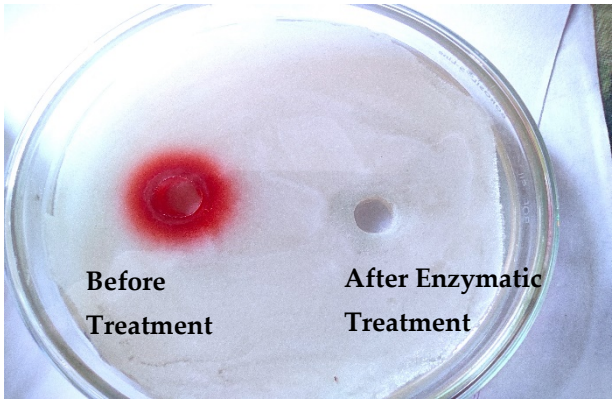


Fig. 10: Microbial Study

4. CONCLUSIONS

Peroxidase extracted from Macrotyloma uniflorum was successfully immobilized on PVA-chitosan beads via crosslinking, retaining high activity and enhanced stability compared to the free enzyme. The immobilized enzyme enabled efficient and nearly complete degradation of Congo red in the presence of H_2O_2, maintaining activity over eight reuse cycles. This method offers a cost-effective, straightforward, and eco-friendly approach for the bioremediation of Congo red, with practical potential for application in dye effluent treatment of wastewater from the dye industry. Potential scalability of this bioremediation approach for industrial applications is promising due to its high enzyme retention (96%), rapid degradation efficiency (100% decolorization in 10 minutes), and reusability over multiple cycles. The industrial-scale application of this approach remains a subject for future investigation.

Author Contributions:

Neha Bhatkar – Writing- original draft preparation

Aparna Kulkarni – Conceptualization and supervision

Seema Devasthali – Visualization and formal analysis

Varsha Ghadyale – Methodology

All authors have read and agreed to the published version of the manuscript." Authorship should be restricted to individuals who have made significant contributions to the research.

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Conflicts of Interest: The authors declare no conflicts of interest.

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