Uttar Pradesh Journal of Zoology

Volume 45, Issue 16, Page 189-197, 2024; Article no.UPJOZ.3786
ISSN: 0256-971X (P)

Integrated Enzymatic Bioremediation: Efficient Decolorization of Textile DYE Effluents and Assessing Environmental Safety

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.56557/upjoz/2024/v45i164299

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/3786

Original Research Article

Received: 16/05/2024
Accepted: 20/07/2024
Published: 27/07/2024

ABSTRACT

Background: The synthetic dyes have become a staple in various industries, as colors play an important role in consumer choices. However, these dyes pose various health and environmental risks.
Aim: The study aimed to identify bacterial isolates capable of producing enzymes for dye decolorization and assess their effectiveness in removing synthetic dyes. Additionally, the study sought to evaluate the toxicity levels of decolorized water samples, contributing to the development of eco-friendly dye removal technologies.

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Cite as: *Umamaheswari, K., and R.K. Sumathi. 2024. "Integrated Enzymatic Bioremediation: Efficient Decolorization of Textile DYE Effluents and Assessing Environmental Safety". UTTAR PRADESH JOURNAL OF ZOOLOGY 45 (16):189-97. https://doi.org/10.56557/upjoz/2024/v45i164299.*

Methodology: Bacterial isolates were obtained from textile effluent samples and screened for their ability to degrade synthetic dyes and produce enzymes. Enzyme production was evaluated through specific assays, and decolorization efficiency was determined using spectrophotometric analysis. Toxicity tests were conducted to assess the safety of the decolorized solutions. Furthermore potential isolate was subjected to 16sr RNA sequence analysis for species level identification.
Results: The study isolated bacterial strains capable of producing lipase, amylase, cellulase, and laccase enzymes, with the majority of isolates (48%) exhibiting laccase production. One isolate, isolate 19 wasproducing laccase was demonstrated high decolorization efficiency for various synthetic dyes, comparable to the efficiency of enzyme producing isolates. Furthermore, the toxicity assay indicated that the decolorized solution using the laccase enzyme was non-toxic, while the isolate-treated solution showed toxicity. Additionally, potential isolate was identified as *Achromobacter xylosoxidans* with 16sr RNA sequence.
Implications: The findings suggest that bacterial isolates have the potential to effectively decolorize synthetic dyes and produce non-toxic decolorized solutions. This research offers eco-friendly solutions for dye removal, addressing the need for sustainable water treatment and minimizing the environmental impact of dyeing processes.

Keywords: Achromobacter xylosoxidans; laccase; decolorization; toxicity; textile wastewater.

1. INTRODUCTION

Wastewater originating from the textile industry emerges as a prominent source of industrial pollution, primarily due to the presence of dyes. With over 10,000 distinct synthetic dyes extensively utilized in textile dyeing and printing processes, these compounds often belong to categories such as heterocyclic, anthraquinone, azo, phthalocyanine, and triphenylmethane. These dye types persist in wastewater and the surrounding environment [1]. The introduction of these dyes into wastewater poses a threat to aquatic ecosystems, as they can disrupt photosynthesis and reduce the solubility of oxygen in water. Consequently, both chemical and biological oxygen demands escalate, leading to an imbalance in the ecosystem [2,3]. Textile industry effluents also contain challenging-to-remove toxic compounds, including heavy metals.

Numerous studies have explored methods to control dye pollution, encompassing physical and chemical treatments such as adsorption, oxidation, and Fenton reactions [4]. While some of these methods may be costly and generate by products requiring further processing, adsorption processes, in particular, are considered incomplete solutions as pollutants are transferred rather than eliminated. Consequently, there is a growing exploration of biological treatments, involving the use of microorganisms for the degradation of synthetic dyes, as viable and cost-effective alternatives [3].

Microorganisms achieve the decolorization of textile dyes through two primary mechanisms: either by adsorption onto microbial biomass or through the biodegradation of dyes facilitated by cells or enzymes. The utilization of biomass is particularly advantageous when the effluent is highly toxic and does not support the growth and sustenance of microbial cells. However, this process may lead to the formation of toxic by-products [5]. Therefore, it is crucial to assess the degraded products when using enzymes to ensure that this negative outcome does not occur.

According to Iark et al. [6], the biodegradation of Congo Red (CR) by laccase leads to the formation of an open benzene ring intermediate product with reduced toxicity. Similarly early study was observed a comparable reduction in toxicity following the degradation of synthetic dyes such as Acid Orange 67, Disperse Yellow 79, Basic Yellow 28, Basic Red 18, Direct Yellow 107, and Direct Black 166 [7]. This suggests that ligninolytic enzymes have the capacity to both degrade and detoxify synthetic dyes. Based on these studies, the present study focuses on assessing the production of lipase, amylase, cellulase, and laccase enzymes, along with the discoloration of the synthetic dyes. Bacteria isolated from samples of a textile wastewater treatment plant are employed, paving the way for future bioremediation studies targeting industrial effluents.

2. MATERIALS AND METHODS

The effluent collected from textile industries located in Erode and Pallipalayam was stored in clean plastic cans at 4°C for subsequent

physiochemical analysis. Water samples were obtained from the industry outlet at a river point, using sterile 500 mL glass vials. At the time of collection, the pH and temperature of the sample were analyzed using pH tape and a thermometer, respectively.

2.1 Isolation of Bacterial Isolates from Textile Effluent Samples

For bacterial isolation, the collected sample underwent serial dilution (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). Aliquots of 100 µL from each dilution were then spread into Nutrient Agar (NA) culture media for bacteria isolation. The plates were incubated at 37°C for 24 hours, and bacterial colonies were observed. The isolated bacteria were preserved at -20°C with 20% glycerol [8].

2.2 Screening of Dye Degrading Isolates

In this study, commonly used azo dyes in textile industries were employed. The dyes were dissolved in nutrient broth at a concentration of 100 mg L-1 and dispensed into screw cap test tubes (10 mL per test tube). Subsequently, these tubes were inoculated with isolated test organisms individually and incubated at 35°C for 3 days under static conditions. After the incubation period, the isolates exhibited promising prospects for dye decolorizationin the test tubes, as observed [8].

2.3 Screening of Laccase Producing Isolates

For the evaluation of ligninolytic enzymes production, the bacteria were isolated from textile effluent as previously described, the Luria Bertani (LB) with the addition of Guaiacol (0.01%). Production of a brownish color around bacterial isolates was considered positive for ligninolytic activity [9].

2.4 Screening of Cellulase Producing Isolates

To screen for cellulase production, isolates were cultured on plates supplemented with 10 g/l carboxymethyl cellulose (CMC). Following 48 hours of incubation at 30°C, the presence of clear halos around colonies was observed, signifying cellulolytic activity [10].

2.5 Screening of Amylase Producing Isolates

Bacterial isolates were spot-inoculated on starch agar plates, and subsequent incubation at 30°C for 48 hours revealed the development of clear zones around colonies upon flooding with iodine solution. This observation indicated positive results for amylase production, as followed by Senthamil and Thangaraj [10].

2.6 Screening of Lipase Producing Isolates

Bacterial strains were isolated and subjected to screening for lipolytic activity using the Tributyrin Agar plate assay method (TBA). The composition of tributyrin agar medium is (per liter) 5 g peptone, 3 g yeast extract and incorporating 1.0% (v/v) olive oil and subsequently sterilized at 121 °C for 15 minutes. After sterilization, the media were poured into petri plates. The isolated strains were streaked onto the Tributyrin agar plates, followed by incubation at 37 °C for 24 hours to assess the development of observable zones [11].

2.7 Assay for Laccase Enzyme

The laccase assay for guaiacol oxidation, as reported by Kuntal et al. [12], involves the development of a reddish-brown color due to the oxidation of guaiacol by laccase, with enzyme activity measured at 450 nm. The reaction mixture is prepared as follows:

(a) Guaiacol (2 mM) - 1 ml
(b) Sodium acetate buffer (10 mM) - 3 ml
(c) Enzyme source - 1 ml (fungal supernatant)

A blank, serving as a control, is prepared with 1 ml of distilled water instead of enzyme. The mixture is then incubated at 30 °C for 15 minutes, and the absorbance is read at 450 nm using a UV spectrophotometer.

Enzyme activity is quantified in International Units (IU), where 1 IU represents the amount of enzyme required to oxidize 1 µmol of guaiacol per minute. The laccase activity in U/ml is calculated using the following formula: [Provide the formula for calculating laccase activity in U/ml here].

$$E.A = A \times V/t \times e \times v$$

Where E.A = Enzyme activity A = Absorbance, V = Total mixture volume (ml), v = enzyme volume (ml), t = incubation time, e = extinction coefficient for guaiacol (0.6740 lM/ cm).

2.8 Decolorization of Dye with Enzyme

The oxidative potential of the ammonium sulphate precipitatedlaccase for dye removal was evaluated on five synthetic dyes following a standard technique [13]. The dyes were Methylene Blue, Congo Red, Coomassie Brilliant Blue, phenol red andbromothymol blue. About 100 mg/L of each dye was dissolved in sterile tap water. Ten unit of ammonium sulphate precipitated enzyme was mixed with 1 ml of phosphate buffer saline and each dye solution was allowed to react at a 1:1 ratio. The mixture was incubated at 30 °C for 24 hrs. The procedure was repeated for all five dyes, and the absorbance was read, respectively, at 595nm using a colorimeter. Decolorization rate (%) = (A0 − A1) A0 × 100 (1), A0 is the dye absorbance before decolorization and A1 is the dye absorbance after decolorization.

2.9 Decolorization of Dye with Isolates

The above mentioned dyes and same concentrationwas prepared in nutrient broth and 10% of inoculumswereadded and incubated for 24hrs. Decolorization efficiency was analyzed by measuring the absorbance of culture supernatant at 484 nm. The decolorizing efficiency was expressed as percentage of decolorization [14] with above mentioned formula.

2.10 Determination of Toxicity

The bioassay aimed to assess the inhibition of bacterial growth by degraded components in the decolorized media of five dyes. This evaluation was conducted using a clear supernatant obtained after bacterial and laccasedecolorization, comparing it with the toxicity of the media before treatment (used as control samples). The procedure involved incubating *E. coli* [15] at 35°C and 150 rpm in nutrient broth. After 24 hours, 2 ml of the bacterial culture was transferred onto sterile Petri dishes containing nutrient agar. Following an additional 24 hours, holes were punctured in the seeded Petri dishes, and 50 µl of the clear supernatant from non-treated, isolate-treated, and laccase-treated samples were added to the respective holes. After an additional 24 hours, the diameter of the inhibition zone was measured.

2.11 16S rRNA Gene Sequence

The potential isolate (the one exhibiting the highest enzyme activity) was identified to the species level using its 16S rRNA gene sequence. This sequence underwent BLAST analysis against the NCBI GenBank database, selecting the top ten sequences with the highest identity scores. These sequences were aligned using Clustal W, followed by the generation of a distance matrix and construction of a phylogenetic tree using MEGA 7.

3. RESULTS AND DISCUSSION

In order to isolate dye decolorizing bacteria, the enriched culture broth were inoculated in nutrient agar medium amended with the dye mixture. A total of 25 morphologically distinct bacterial colonies were isolated and screened out by repeated streaking on dye supplemented nutrient agar medium. All isolates were subjected to decolorization analysis against five commercial dyes.

The results of the dye degradation study reveal varying degrees of effectiveness among the different isolates in decolorizing the tested dyes. To identify the isolates with the highest percentage of decolorization for each dye, it is necessary to consider the instances where a particular isolate demonstrated positive degradation. The highest percentage of decolorization is indicative of an isolate's superior ability to break down and remove the respective dye from the medium. Among the 5 types of dye utilized, Methylene Blue was highest decolorized than other dyes.

In the present study, single Isolate was consistently displayed positive degradation for all tested dyes. Consequently, IS19 is likely to have the highest percentage of decolorization for each of these dyes. Another isolate also demonstrated positive degradation for all dyes, except Coomassie Brilliant Blue, indicating a high potential for decolorization. Additionally, among the 25 isolates, 5 of were showed positive degradation for multiple dyes, suggesting significant decolorization capabilities.

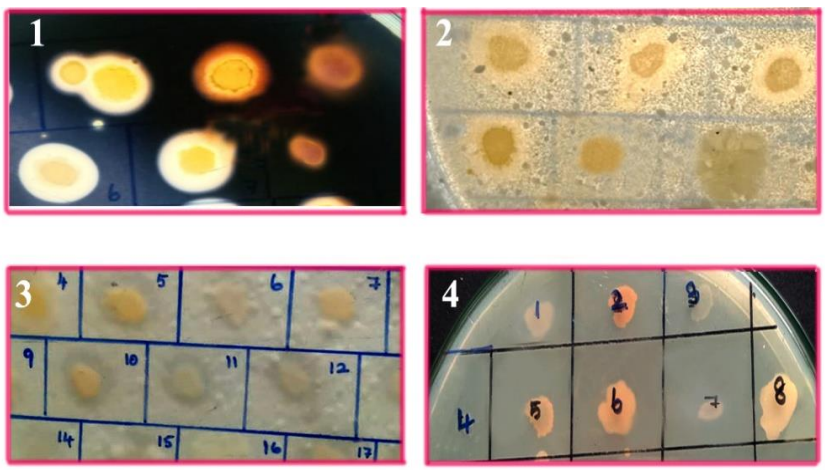
In earlier investigations, numerous researchers identified potential dye-decolorizing bacterial isolates sourced from various environments, including textile dye effluent, activated sludge; soil contaminated with dye from waste disposal sites, lake mud, and wastewater treatment plants [16,17,18] and [8]. These findings underscore the natural adaptation of these isolates to elevated dye concentrations and their ability to survive in the presence of hazardous chemicals. However,

no one in this area has done so far to remove the various dyes with the microbes that depend on their habitat, especially within 48 hours.

Numerous studies have been published on dye removal by fungi, particularly focusing on the decolorization of dyes through enzymes produced by fungi. Extracellular oxidoreductases such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase have been extensively investigated for their ability to efficiently decolorize dyes [5]. In case of bacteria, that there is a limited body of research on the decolorization of dyes by enzymes other than laccase, particularly those produced by bacteria, previous study of Bernal et al. [19] were determine the various decolorizing enzyme. In the current study, the emphasis is placed on evaluating the decolorizing potential of enzymes laccase and other than laccase, specifically lipase, amylase, and cellulase, within isolates sourced from textile dye-containing environments. The majority of isolates (48%) exhibited laccase production, followed by lipase (36%) and cellulase (24%) (Fig. 1). The observed trend in these results aligns with the findings of a prior study by Yuxian et al. [20], where they similarly noted the predominance of laccase production in fungal isolates.

Among the 25 isolates tested, 5 were capable of producing all four enzymes. These findings align with the results of dye decolorization, where isolates with the potential to produce multiple enzymes demonstrated effective decolorization across all tested dyes. The co-occurrence of all enzymes within these isolates suggests a potentially heightened capacity for dye removal. This phenomenon was similar to previous study of Yuxian et al. [20]. In this study, a majority of the isolates produced laccase; hence, laccase was chosen to assess the efficiency of dye decolorization. The efficacies of dye decolorization using laccase were tested across Methylene Blue, Congo Red, Coomassie Brilliant Blue, Phenol Red, and Bromothymol Blue. Isolate 19, which produces laccase, exhibited the highest decolorization percentages: 83.5% for Methylene Blue, 71.4% for Congo Red, 69.5% for Coomassie Brilliant Blue, 82.7% for Phenol Red, and 60.7% for Bromothymol Blue (Table 1). Isolate 14, also producing the enzyme, showed strong decolorization, particularly with Methylene Blue (80.2%).

While there are limited studies on the removal of dye using the laccase enzyme, a majority of the research has focused on laccase-producing bacteria. However, in Kangli et al. [21] employed the laccase enzyme to decolorize methylene blue. Previous study of Navleen et al. [22] also decolorized the 10Unit of laccase for Indigo carmine. Latterly, [13] was decolorize the various dye including Congo red and other dyes with Bacillus producing laccase enzyme. Number of authors reported that fungal laccase was utilized for decolorized the various textile, however fungal laccase not tolerate the high temperature and pH environments [22].



1, 2 and 3 :Screening of Amylase, Cellulase and lipase producing isolates
Positive isolates identified by zone of inhibition around the colonies
4. Screening of laccase producing isolates with identified by brown colonies

Fig. 1. Screening of enzyme producing isolates

Table 1. Efficacy of dye decolorization with laccase enzyme

		% of Dye decolorization				
S. no	**Isolates**	**Methylene Blue**	**Congo Red**	**Coomassie Brilliant Blue**	**Phenol Red**	**Bromothymol blue**
1.	Is 4	65.9	38	49.5	58.6	50.6
2.	Is 7	64.8	57.1	37.1	51.7	48.1
3.	Is 14	80.2	38	46.6	58.6	50.6
4.	Is 19	83.5	71.4	69.5	82.7	60.7
5.	Is 21	76.9	47.6	44.7	65.5	54.4

In this study, besides employing the laccase enzyme for the decolorization process, the isolates that produced the enzyme were also utilized to assess decolorization efficiency. Notably, IS19 demonstrated the highest capability, achieving a decolorization rate of 77.7% for methylene blue, 65.4% for Congo Red (CR), 43.4% for Coomassie Brilliant Blue (CBB), 57.1% for phenol red (PR), and 42.5% for Bromothymol Blue (BTB) (Table 2). In recently [23] also determine the methylene blue degrading bacterial isolates from dye contamination area. Other hands [24,25] were also observed the congo red decolorized bacterial isolates (Fig. 2).

Table 2. Efficacy of dye decolorization with bacterial isolates

		% of Dye decolorization				
S. no	**Isolates**	**Methylene Blue**	**Congo Red**	**Coomassie Brilliant Blue**	**Phenol Red**	**Bromothymol blue**
1.	Is 4	48.1	29.4	22.2	39.2	27.5
2.	Is 7	44.3	41.1	17.1	50	31.2
3.	Is 14	49.3	23.5	21.2	35.7	40
4.	Is 19	77.7	65.4	43.4	57.1	42.5
5.	Is 21	60.4	41.1	30.3	32.1	30

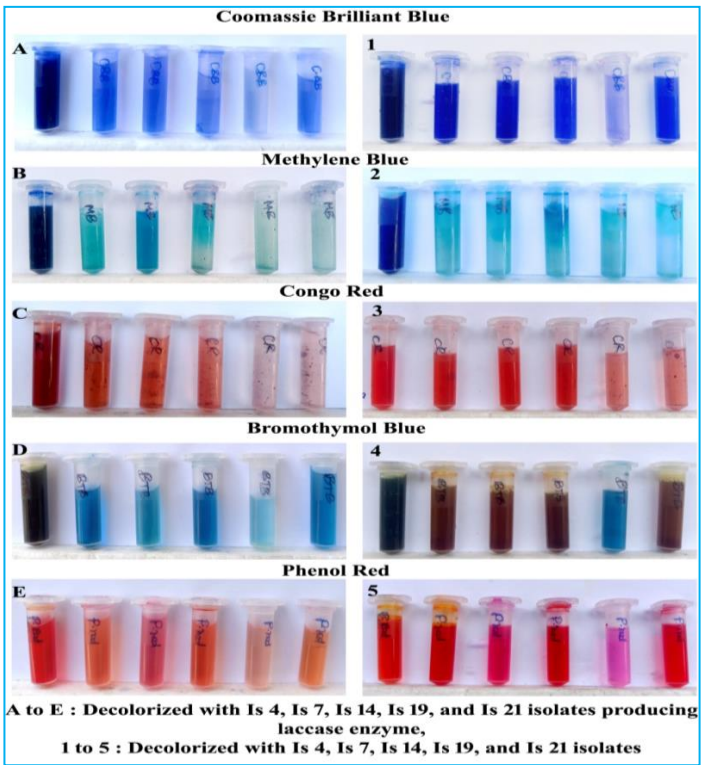


Fig. 2. Decolorization of various dye with enzyme and isolates

This study observed some significant variation in decolorization levels when using laccase and isolates. Nonetheless, given that numerous studies have reported the generation of toxins during bacterial decolorization [22], our investigation aimed to determine the toxicity levels associated with the decolorized enzyme or its producing isolates from water samples subjected to decolorization. In this study, isolates and it producing laccasewere selected to toxicity assay. Toxicity tests confirm the safety of treated dye resulted from biological treatment by isolate and enzyme. The result was showed that there is no inhibition zones are formed while using laccase enzyme except Is 21, which indicating the lack of toxicity of the solution, simultaneously, zone of inhibition was appeared on decolorized water with using isolates (Is4 and Is 21), which indicate as toxicity present in the decolorized solution (Fig. 3).

3.1 2-Determination of Toxicity of Laccase Producing Isolates

According to literature, no one was reported that similar line of results, however, previous authors reported that toxin producing materials were observed while decolorized with isolates [7]. In recent study of Chiedu [13] were observed the lack of toxicity while using the laccase during the decolorization. The another hand of Liu et al. [25] also determine the less toxic effect on congo red decolorized solution with fungal isolate producing ligninolytic enzymes, they were proofed with phytotoxicity assay. From these statements it is clear that the laccase enzyme produced by bacteria or fungi is formed during the decolorization with non-toxins and low-toxins.

Based on nucleotide homology and phylogenetic analysis, isolate 19 was identified as *Achromobacter xylosoxidans* strain, demonstrating a 100% match over 2540 nucleotides with accession number MN394127.1 in the NCBI database. Further characterization through 16S rRNA gene sequence analysis, followed by BLAST analysis and phylogenetic tree construction using MEGA11, corroborated these findings. The phylogenetic tree confirmed the close relationship between the isolate and *Achromobacter xylosoxidans*, substantiating its identification (Fig. 4).

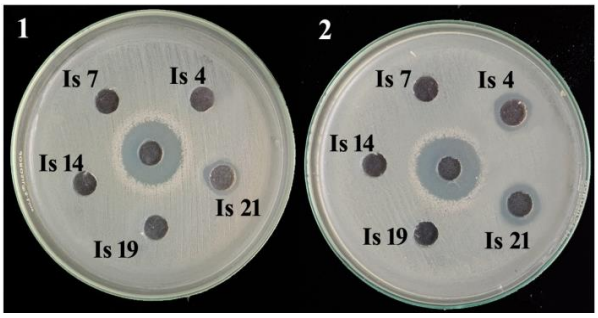


Fig. 3. Determination of toxicity: 1-Toxicity determination with laccase

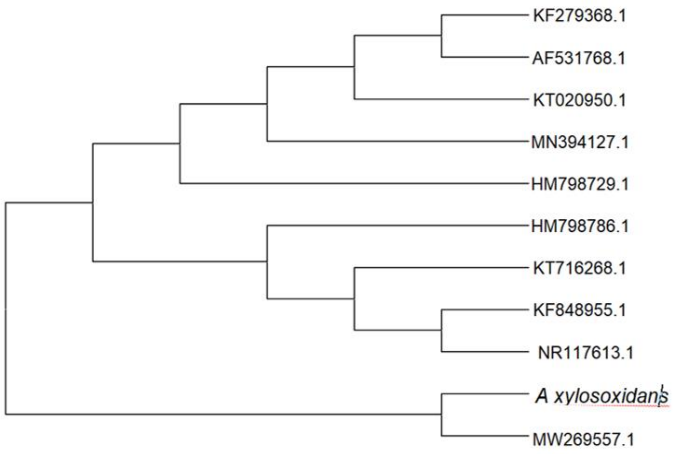


Fig. 4. Phylogentic analysis of *Achromobacter xylosoxidans*

4. CONCLUSION

This study successfully isolated dye-decolorizing bacteria, emphasizing laccase, lipase, amylase, and cellulase production. The prevalence of laccase-producing isolates, notably IS19, displayed consistent positive degradation and remarkable decolorization efficiency for various dyes, including Methylene Blue, Congo Red, Coomassie Brilliant Blue, phenol Red, and Bromothymol Blue. The research's novelty lies in evaluating multiple enzymes, selecting IS19 for decolorization, and exploring safety through toxicity assays. These findings contribute to understanding bacterial adaptation to dye-rich environments and offer eco-friendly solutions for dye removal. The observed lack of toxicity with laccase suggests potential for non-toxic water treatment methods. The study's societal impact includes the development of rapid, environmentally friendly dye removal technologies, addressing the need for sustainable water treatment and minimizing the environmental impact of dyeing processes. Overall, this research advances scientific knowledge and holds promise for practical, sustainable applications benefiting society and the environment.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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DOI: 10.1098/rsos.200688.

Peer-review history:
The peer review history for this paper can be accessed here:
https://prh.mbimph.com/review-history/3786