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Decolorization of azo dye Congo red by manganese dependent peroxidase of immobilized *Pleurotus sajor caju*

Ramy S. Yehia[1]* and Hibah I Almustafa[1]
[1]Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa 31982, Saudi Arabia
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*to whom all correspondence should be addressed: e-mail: ryehia@kfu.edu.sa
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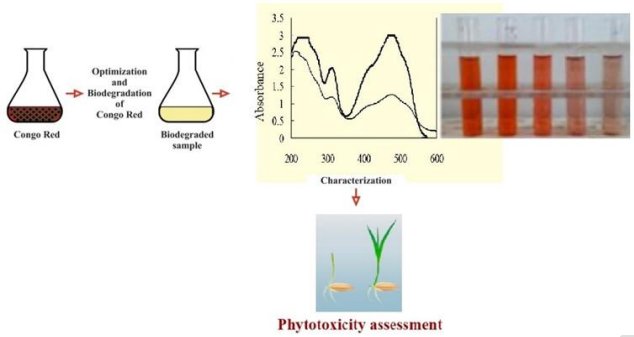
Graphical abstract



Abstract

Azo dyes and the byproducts of their degradation have the potential to pollute the environment. Natural water bodies experience less sunlight penetration as a result of these effluents being released into rivers and lakes. The decolorization potential of an enzyme generated by a local white rot fungus is described in this research. Using an economical medium that includes agro-industrial residue in solid-state fermentation (SSF), the fungus *Pleurotus sajor caju* was able to optimize its production of ligninolytic enzymes. Comparing to the peels of mandarins, oranges, and cantaloupes, banana peels were the most effective substrate for the formation of extracellular manganese dependent peroxidase (MnP). During the ten days, the highest activity of 6.3 U ml^{-1} was observed. After being immobilized, the enzyme was used to decolorize Congo Red (CR) dye. For maximum decolorization, the optimal pH, temperature, and enzyme concentration were determined to be 4, 35°C and 1.4 U ml^{-1}, respectively. Manganese-dependent peroxidase that had been partially purified shown remarkable decolorization activity to CR. The highest percentage of decolorization (95%) was noted. The UV-Vis spectrometer was used to track the biodegradation of CR. Following MnP treatment, CR's phytotoxicity (with regard to *Sorghum vulgare* and *Phaseolus radiatus*) was frequently decreased. As a result, this work may serve as a framework for the larger-scale manufacture and use of MnP on an inexpensive substrate. To the best of our knowledge, this is the first report of MnP effectively detoxifying and decolorizing azo dye from immobilized *P. sajor caju*.

Keywords: Phytotoxcicity, Biodegradation, *Pleurotus sajor caju*, Azo dyes, Immobilization, solid-state fermentation

Highlights

Cost-effective strategy for Congo Red bioremediation.

MnP efficiently decolorize azo Congo Red dye.

MnP treated dye significantly reduced the phytotoxicity

1. Introduction

The necessity of selecting innovative molecules that can be utilized in a variety of industrial processes is growing along with the scientific and technological development (Rauf and Ashraf 2009). One of the main issues facing the modern world is the increasing environmental degradation brought on by rapid industrial expansion (Merabet *et al.* 2009). Wastewater containing industrial dyes is discharged into the environment. The use of more than 10,000 synthetic dyes as colorants in the food, pharmaceutical, plastic, photographic, paper, petroleum, cosmetic, textile, and leather industries is growing. These dyes pose major health risks to all members of the ecosystem, particularly to humans and animals (Rodriguez 2008; Prajul *et al.* 2025). It is thought that several of them are carcinogenic and hazardous (Hamedaani *et al.* 2007).

Anthracene, benzene, naphthalene, toluene, and xylene are examples of coal-tar-based hydrocarbons that may be the source of dyes' synthetic origin, various molecular weights, and complicated aromatic structures (Knapp *et al.* 1995; Wong and Yu 1999). About 60% of dyes generated belong to the azo group, which is also known as monoazo, diazo, and triazo dyes (Ayed *et al.* 2011) and the most frequently released group into the environment (Ertugrul *et al.* 2008; Venkatraman *et al.* 2025). Dye classifications depend on the chemical structure of chromogenic groups and include azo, heterocyclic polymers, anthraquinone, and triphenylmethanes (Maalej-Kammoun *et al.* 2009; Yang *et al.* 2009). Consequently, these colors must be eliminated before the effluents are released into the environment.

Adsorption, filtration, neutralization, precipitation, chemical or photo degradation, ozone oxidation, and coagulation are just a few of the physical or chemical processes that can degrade and decolorize synthetic dyes. Additionally, these treatments have certain drawbacks, such as being expensive, unappealing to the market, and requiring the disposal of huge amounts of sludge with hazardous byproducts (Konsowa 2003; Binupriya *et al.* 2007, 2008; Sathishkumar *et al.* 2012; Suresh *et al.* 2025). Colorless aromatic amines, which are typically more toxic than the original compounds (Kulla *et al.* 1983; Banat *et al.* 1996), may be produced when bacteria are used in the biotreatment of dye effluents. As a result, they may not be as adaptable and may only be used for a limited variety of dye wastewater (Kulla *et al.* 1983).

Specifically, fungi have the ability to decolorize textile effluent by the oxidative destruction of dye molecules or the adsorption of pigment on fungal mycelium (Fu and Viraraghavan 2001). White rot fungi are appealing organisms for use in pollutant site sanitation because they produce a lot of extracellular lignin-modifying enzymes (Arora and Sharma 2010), which show potential in the aerobic degradation and detoxification of a variety of xenobiotic compounds (Kumar *et al.* 2007) (Mohorcic *et al.* 2006). The ligninolytic enzymes, including laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), and manganese-dependent peroxidase (EC 1.11.1.13), are the most significant enzymes involved in the decolorization of dyes.

These enzymes are capable of attacking a wide range of complex aromatic dyestuffs (Kamitsuji *et al.* 2005) and are primarily thought to form during secondary metabolism of white rot fungi based on nutrient limitation, either nitrogen or carbon, rather than the presence of pollutant (Hou *et al.* 2003). The natural habitats of white rot fungus are not replicated by submerged fermentation. One advantage of solid state fermentation (SSF), which is the fermentation of solids without the presence of free water, is that it promotes the development and metabolism of microorganisms in damp environments (Pandey 2003). SSF's ability to produce enzymes from agricultural waste has drawn a lot of interest in the biotechnology community because of its low production costs and increased productivity (Pandey 2000).

In addition to supplying comparable substrates, the utilization of such wastes aids in resolving environmental issues brought on by their disposal. Furthermore, the majority of these wastes include cellulose, hemicellulose, and/or lignin, which serve as mediators of the lignolytic activities. Additionally, the majority of them are rich in sugar, which greatly reduces the cost of the operation. Because of all these factors, they are highly advantageous as raw materials for microbes to produce secondary metabolites of their commercial significance (Kalogeris *et al.* 2003).

This is the first investigation on the immobilization of *Pleurotus sajor caju* into Ca-alginate beads, producing manganese dependent peroxidase for its use in dye decolorization. The current study intends to examine the manganese dependent peroxidase produced from the potential alginate-immobilized Pleurotus sajor caju under SSF for decolorization of commercial azo dye.

2. Materials and Methods

2.1. Microorganism and growth medium

P. sajor caju was kindly provided by National Research Center, Giza. Egypt. The fungus was maintained on potato dextrose agar plates at 28±2°C for 7–8 days. The organism was then kept at 4°C for future use.

2.2. Agro-industrial wastes preparation

Under solid state fermentation (SSF) conditions, chopped banana (*Musa cavendishii*), orange (*Citrus sinensis*), mango (*Mangifera indica*), and cantaloupe (*Cucumis melo*) peels (particle size 7.5 mm × 7.5 mm) were purchased from the local market and utilized as a substrate for *P. sajor caju* to produce MnP. The substrates underwent the following pre-treatment: To neutralize organic acids, 10 g of each freshly collected substrate was initially soaked in 30 ml of 83.17 mM KOH at room temperature for an hour (Stredansky and Conti 1999). They were subsequently dried at 50°C after being carefully cleaned with distilled water.

2.3. Immobilization of pleurotus sajor caju

With minor adjustments, *P. sajor caju* was trapped in Ca-alginate polymer beads using a technique outlined by Laca *et al.* (2000). 100 ml of malt extract media (pH 6.5) were used to cultivate *P. sajor caju*. The mycelial pellets were cleaned with sterile phosphate buffer (pH 7.0) after five days, and the mycelial mat was then ground with acid-washed sand using a pestle and mortar to homogenize it. 100 ml of 2.1% (w/v) sodium alginate were shaken with five ml of mycelial mat. Alginate's ultimate concentration in the beads was 2%. Using a syringe, the mixture was injected into a 3% (w/v) $CaCl_2$ solution while being shaken.

The beads were removed from the solution after 30 min, cleaned with distilled water, and then left in a 0.5% (w/v) sodium alginate solution for 5–10 min. The beads were then rinsed with purified water and allowed to solidify for half an hour in a 3% (w/v) $CaCl_2$ solution. Furthermore, 0.7% NaCl was used to wash the beads. Every solution in issue was autoclaved before being used.

2.4. Enzyme preparation

Four distinct agro-industrial wastes, including orange peel (OP), banana peel (BP), mango peel (MP), and cantaloupe peel (CP), were used as the substrate in the manganese-dependent peroxidase manufacturing process in SSF. Additionally, 10 grams of moistened peels were placed into a 250 ml conical flask and autoclaved for 15 minutes at 121°C. Each flask was further enriched with 100 µM $MnSO_4$ as a promoter for the production of enzymes, and 50 immobilized beads (4 mm approximately) of the mycelial mat trapped in alginate were added. The flasks were then allowed to incubate at room temperature. Cultures were taken out every two days to measure the amount of extracellular MnP enzyme.

The culture was soaked in 100 mM sodium acetate buffer pH 5.0 at 4°C for the entire night in order to extract MnP. After filtering and centrifuging the culture supernatant for 15 min at 10,000 × g to eliminate any fine particles, 80%

(w/v) ammonium sulfate was added to precipitate it. The precipitate was recovered by centrifugation at 10,000 × g for 30 minutes after standing in the ammonium sulfate solution for 5 h at 0ºC. It was then resuspended with 100 mM sodium acetate buffer (pH 5.0) and dialyzed against the same buffer overnight at 4°C. The final solution is prepared for research on decolorization.

2.5. MnP assay

By applying the molar extinction coefficient value of 65,00 M-1 cm-1 and tracking the absorbance shift at λ= 240 nm caused by the synthesis of Mn^{+3} lactate, the activity of MnP was ascertained spectrophotometrically (Gold and Glenn 1988). 50 µM $MnSO_4$, 50 µM H_2O_2, and 0.5 ml of the enzyme solution in 50 mM sodium lactate/lactic acid buffer pH 4.5 at 30°C constituted the 1 ml reaction mixture. The quantity of enzyme required to oxidize one µmol of the substrate per minute under the given assay conditions was referred to as one unit of enzyme activity.

2.6. In vitro decolorization of CR by P. sajor caju MnP

CR was used to investigate dye decolorization performance in order to achieve the optimal enzyme activity derived from SSF. The reaction, which involved CR dye, partly purified MnP, and 100 mM buffer, was conducted directly in spectrophotometer cuvettes with a total volume of 1 ml. The reaction mixture was incubated to degrade the dye, and the degree of decolorization was measured using a UV-Vis spectrophotometer to track the absorbance peak at the maximum wavelength of CR dye, 498 nm, and represented as a percentage. For this experiment, heat-denatured MnP served as a control. The following formula was used to determine the rate of CR decolorization:

$$\%D = (A_i\text{-}A_f)/Ai\times100$$

D was the color removal rate, and Ai and Af indicated for the absorbency of the corresponding treated dye and the enzyme-free control, respectively. Each test was carried out twice, and averages were calculated.

2.7. Optimization of decolorization process

Through a fixed dosage of dye (150 ppm) and an enzyme concentration (2 U ml^{-1}) at 28°C ±2 with the appropriate buffer present, the impact of pH values that varied from 3 to 10 on enzymatic decolorization was observed. 50 mM sodium acetate (3–7), 50 mM sodium phosphate (pH 6.0–8.0), and 50 mM Tris-HCl (8–10) were used for modifying the pH of the reaction mixture.

The temperature ranged from 20 to 80°C, with an additional 10°C. The reaction was initiated with varying amounts of enzyme, ranging from 0.2 to 2 U ml^{-1}, with increments of 0.2 U ml^{-1}, in order to determine the enzyme concentration on dye decolorization with a fixed value of dye (150 ppm) and pH (6.5) at 35°C. After 30 min of incubation, the decolorization was examined for each of these tests.

2.8. Estimation of total phenolics

With a few modifications, the total phenolics were identified using Singleton *et al.* (1999) following the entire decolorization of CR and varying enzyme concentrations. The reaction mixture, which included 250 µl of Folin-Ciocalteu reagent, 3 ml of distilled water, partially purified MnP, and dye (150 ppm), was incubated for one min at 30°C. Following incubation, 750 µl of 10% Na2CO3 was added to the reaction mixture, and it was then incubated for 60 min in the dark at 30°C. By measuring absorbance at 760 nm in comparison to the blank, the amount of phenolics was ascertained.

2.9. Phytotoxicity assay

Using the approach of Zucconi *et al.* (1985) with minor changes, phytotoxicity was carried out to evaluate the toxicity of CR and MnP-treated CR solutions on the germination of typical agricultural crops of mung bean (*Phaseolus radiatus*) and sorghum (*Sorghum vulgare*) seeds. The seeds were sterilized for 5–10 minutes in a 5% sodium hypochlorite solution for this experiment (USEPA 1996), and they were then thoroughly soaked many times with deionized water. 1:6 dilutions of dye samples were utilized. After being submerged in CR and MnP-treated CR solutions, ten seeds were left to incubate in a dark environment. After seven days of incubation, the proportion of seeds that germinated as well as the shoot and root elongation of the test sample in each solution were noted.

Deionized water was employed for control. The following formula was utilized to determine the germination index (GI): GI stands for germination index, GP for number of germinated seeds expressed as a percentage of control values, La for average root length in CR solutions, and Lc for average root length in control. Every experiment was carried out three times, and the mean values of the trials were used to express the results.

$$GI= GP \times L_a / L_c$$

2.10. Statistical analysis

The Tukey Kramer multiple comparisons test and one-way analysis of variance (ANOVA) were used to examine the data. When $P \leq 0.05$, the readings were considered significant.

3. Results and discussion

Ca-alginate was utilized as a supporting material to immobilize the white-rot fungus *P. sajor caju*. **Figure 1** shows the manufacture of MnP from immobilized *P. sajor caju* in SSF using inexpensive, locally accessible agro-industrial waste materials, such as banana peel (BP), orange peel (OP), mandarin peel (MP), and cantaloupe peel (CP), in order to lower the cost of the SSF process. After four days of incubation, mycelial development on the wastes was observed, and within seven days, the fungal mycelium had completely colonized the substrate. The production of MnP was examined every two days.

After 12 days of incubation, the maximum MnP activities of immobilized P. sajor caju were found to be 6.3 U ml^{-1} of BP, 5.7 U ml^{-1} of OP, 5.2 U ml^{-1} of MP, and 4.5 U ml^{-1} of CP. Microorganisms may readily consume this because to has a high glucose content (Essien *et al.* 2005). According to Osma *et al.* (2007), under SSF, the white rot fungus *Trametes pubescens* grew and produced laccase on banana

skin, a unique substrate. Immobilized *P. sajor caju* produced a similar result in our work, suggesting that BP can be utilized as an inexpensive source for the manufacture of MnP. For dye decolorization experiments, *P. sajor caju* extracellular MnP made from BP culture substrate was utilized.

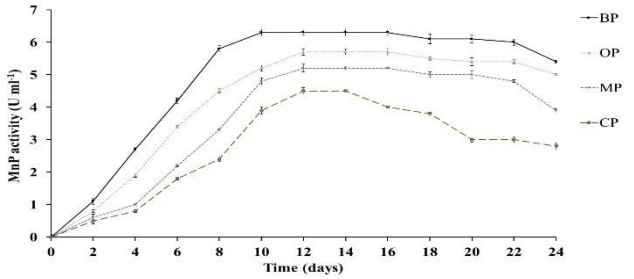


Figure 1. Production of MnP in SSF by immobilized *P. sajor cajo*; (BP) banana peel, (OP) orange peel, (MP) mandarin peel, (CP) cantaloupe peel

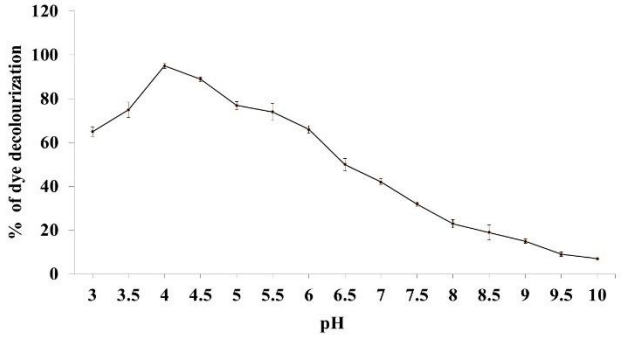


Figure 2. Effect of different pH values on decolorizaion performance of congo red by immobilized *P. sajor caju* MnP.

Figure 2 illustrates that pH, which ranges from 3 to 10, was crucial in the decolorization of CR by crude MnP. According to our findings, pH 4 was the optimal pH for achieving 95% dye decolorization. This result is in line with a prior study that showed that MnP has a pH range of 2.0 to 6.0, with an ideal pH range of 4.0 to 4.5 (Yang *et al.* 2005). At pH 7.5, the decolorization activity dropped, and at alkaline pH, no action was detected. This outcome is consistent with Asgher *et al.* (2009), who reported that at pH 3.0, *Coriolus versicolor* IBL-04 produced the highest amount of MnP-induced ART effluent decolorization (63%) by MnP. *Aspergillus fumigatus* XC6, which was isolated from mildewing rice straw, was shown by Jin *et al.* (2007) to be able to decolorize dye effluent at an acidic pH of 3.0. Additionally, Sharma *et al.* (2009) noted that *P. chrysosporium* decolorized orang II to its maximum extent at pH 5.

Figure 3 illustrates how temperature affects CR decolorization. The outcome clearly demonstrates that 35°C was the ideal temperature for maximal decolorization (95%) before that dropped down significantly. This result is in line with Asgher *et al.* (2008) and Swamy and Ramsay (1999).

One important variable that varies from organism to organism is the incubation temperature; even little variations in temperature can have an impact on growth and, ultimately, the synthesis of enzymes. Therefore, the decolorization mechanism's development and enzyme synthesis may be inhibited by higher temperatures (Babu and Satyanarayana 1995; Bhatti 2007). Additionally, it has been suggested that the chemical structures of dyes may affect the enzyme system that breaks down dyes as well as the pattern of its expression (Boer *et al.* 2004).

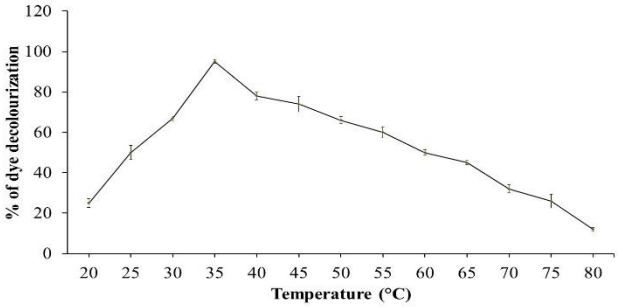


Figure 3. Effect of temperature on decolorizaion performance of congo red by immobilized *P. sajor caju* MnP.

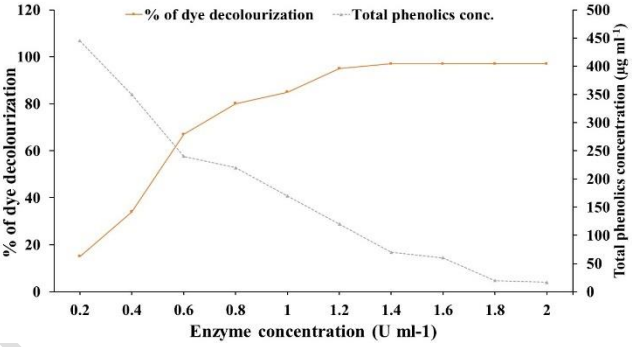


Figure 4. Effect of MnP concentrations on CR-dye decolorization

To establish the amount of enzyme required for maximum decolorization, the effect of enzyme concentration on dye decolorization (150 ppm) was examined. Since 1.4 U ml^{-1} of MnP was the ideal concentration required for maximum decolorization (**Figure 4**), the rate of decolorization improved as the amount of enzyme increased. Additionally, our findings indicate that when enzyme concentration rises, the amount of total phenolics decreases significantly. The amount of phenolics decreased from 445 µg ml^{-1} to less than 20 µg ml^{-1} after decolorization. As a result, this MnP of *P. sajor caju* detoxified these dyes in an environmentally benign way and decomposed similarly without accumulating any phenolics (**Figure 4**).

The ecosystem is seriously contaminated when untreated dyeing effluents are disposed of in water bodies. This water is frequently used for agricultural purposes and has a toxic effect on the biomass and germination rates of many plant species that are essential to ecological functions such as wildlife habitat, soil erosion prevention, and the provision of the majority of organic matter, which is crucial for soil fertility (Kalyani *et al.* 2009; Tan *et al.* 2005; Harazono and Nakamura 2005). According to earlier research, certain azo dyes and the intermediates they produce during decolorization can even cause cancer or mutagenesis (Huang *et al.* 2011).

Therefore, it's critical to assess the phytotoxicity of the metabolites produced throughout the CR decolorization process. The findings of the phytotoxicity investigation are

displayed in **Table 1**, where the sorghum seed's root elongation was 3.92 cm and 100% germination in deionized water was used as a control. When seeds were submerged in CR, the resulting root elongation and seed germination were 1.25 cm and 30%, respectively. Root elongation and seed germination were 3.12 cm and 100%, respectively, in the case of MnP-treated CR.

Additionally, the root length and seed germination of *Phasealus* seeds submerged in CR solution were 0.59 cm and 40%, respectively, whereas MnP-treated CR were 1.59 cm and 100% in contrast to the control, which was 1.64 cm and 100%. *Sorgum* and *Phasealus* seeds submerged in CR and CR solutions treated with MnP had GI values of 32, 80, 36, and 97%, respectively. A GI of less than 50% implies significant phytotoxicity of the effluent, 50–80% suggests moderate phytotoxicity, and >80% shows negligible phytotoxicity, according to Zucconi *et al.* (1985). Therefore, the phytotoxicity of *Sorgum* and *Phasealus* seeds is mild and nonexistent, respectively, for the immobilized *P. sajor caju* MnP-mediated CR degradation products.

Table 1. Phytoxicity study of ifmmobilized MnP from *P. Sajor caju*

Parameter studied	Sorghum vulgare			Phaseolus radiatus		
	control	CR	MnP-CR	control	CR	MnP-CR
Root length	3.92±0.05	1.25±0.07[a]	3.12±0.10[b]	1.64±0.02	0.59±0.15[a]	1.59±0.21[b]
Shoot length	4.56±0.11	2.68±0.09[a]	4.29±0.20[b]	2.48±0.04	1.46±0.017[a]	2.60±0.20[b]
G%	100	30	90	100	40	100
GI	100	32	80	100	36	97

Data presented as mean (±) Standard Error; [a] P≤0.05 vs the control, [b] P≤0.05 vs the CR

G%; seed germination percentage

GI; germination index

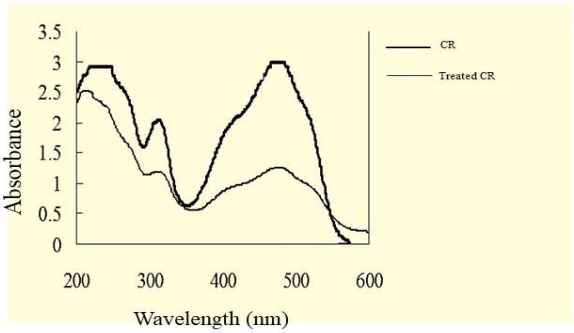


Figure 5. UV–Vis absorption spectrum of congo red decolorization by with or without *P. sajor caju* MnP under pH 4 and 35°C.

Under ideal circumstances, partially pure MnP decolorized CR, and **Figure 5** shows the absorbance spectra. The decolorization of CR was carried out in a UV-Vis spectrophotometer at the previously mentioned ideal conditions, and the findings show a notable transformation following treatment, with the full decolorization of dye visible from the 400–600 nm spectral scan. The chromophoric group's breakdown was suggested by the absorbance peak's disappearance around 498 nm. Interestingly, Congo Red azo dye is promptly decolored by our immobilized MnP derived from *P. sajor caju*.

4. Conclusion

Fungal based methods may also be investigated at industrial scale in mixed culture form and or with other chemical or physical method. Combined technology may reduce the time of treatment required for purely fungal or other biological based methods. Thus, due to the ease of availability of any required amount of fungal biomass, fungal based technology in combined form with other methods of dye treatment may be more efficient in dye polluted wastewater treatment. Consequently, crucial research is being conducted both commercially and in laboratories to address the issue of colorants in effluents using my main approach.

The development of new immobilization supports to decrease enzyme loss and increase enzyme durability; genetic engineering of microbial strains with improved biodegradation characteristics; and additional investigation into the viability of using MnP-assisted decolorization in the large-scale treatment of actual dye wastewater are the main areas of future research.

Conflict of interest

Authors don't have any conflict of interest

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