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Optimization of Various Factors Affecting *Pseudomonas aeruginosa* Mediated Removal of Azo Dye

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Abstract: Due to the depletion of water supplies and heavy environmental pollution, water treatment (WT) is currently one of the main research areas. This has forced the upgrading of conventional technologies towards cheaper, safe, recyclable and reusable side. The objective of this review is to investigate the optimum conditions for the decomposition of methyl red using *Pseudomonas aeruginosa (P. aeruginosa)* to look its usage in surface water purification, since an adequate supply of clean water is necessary for household needs, environmental safety, and health security. The current research illustrates that bacterial populations are effective in decomposition of dye at a particular concentration of dye. In addition, the maximum decomposition was occurred using temperature 37 °C, pH 9, 1400 mg/l of glucose, 1000 mg/l of urea. The research presented here evidently demonstrates that *P. aeruginosa* could potentially be applied to remove color contaminants from textile effluents. Therefore, this specific strain of bacteria could be used for the biological removal of dyeing factory contaminants.

Index Terms **-** Azo-Dyes, Toxicity, Treatment, *P. aeruginosa*, Biological Degradation.

1. Introduction

Dyes have been already reported to inhibit sunlight, reduction in photosynthesis, as well as diminishing the mobility of oxygen. Hence, the dyes are considered to be the primary contaminants in water bodies (Ardila-Leal et al., 2021). Main reasons behind the popularity of Azo dyes in manufacturing industry include their low price, stability, simplicity to be produced, and availability of color's varieties as compared to natural colorants (Slama et al., 2021). Azo dyes are documented to be hazardous, as well as resistant to both heat- and light. In addition, they are highly durable in both acid as well as alkaline conditions, and are found to be non-biodegradable (Oyetade et al., 2022). They also have the capability to persist and accumulate at high concentrations within the surroundings (Ngo and Tischler, 2022). Hence, contamination of water bodies with synthetic dyes could be a risk to the environment as well as human health because of it's toxicity, carcinogenicity, and mutagenicity. Further, ever-increasing statutory restrictions are needed for regulation of effluent discharges (Alsukaibi, 2022). Cancer and carcinogens are among the crucial causes of death of people all over the world (Anwar et al., 2020).

By removal of azo dyes, the current methods—which primarily involve physicochemical processes—take color out of the contaminants found in the textile sector. Treatment techniques for wastewater and effluents include a range of chemical and physical approaches. In addition to using chemicals in the treatment process, these procedures are expensive, produce heavy sludge, and create long-term pollution problems (Samsami et al., 2020).

Scholars are searching for new and inventive methods to eliminate dye from effluents due to above mentioned disadvantages. The exorbitant cost of these outdated physico-chemical processes, secondary contamination from chemical abuse, and poor outcomes when it comes to a large range of hues make them uncommon. The strikingly low effectiveness, cheap prices, simplicity of employing, and ecological sustainability of biological treatments centered around green chemistry allow for a look at viable substitutes. Bio-friendly techniques have been given precedence over physical and chemical techniques in the treatment of wastewater polluted with dyes because of its increased viability, environmental tolerance, and decreased generation of hazardous metabolites (Kumari et al., 2023).

Numerous microorganisms from various taxonomic families, such as bacteria, yeast, and fungi, have proven color decolorization through bio adsorption, biotransformation, or degradation. Due to their ability to grow fast and high speed of degradation, multiple bacteria have been investigated for their ability to break down azo dyes. It is notable that many of these bacteria could generate colorless aromatic amines that are both mutagenic and carcinogenic. (Ngo and Tischler, 2022).

In contrast, certain bacterial cultures may produce aromatic amines and would decrease azo derivatives due to the presence of azo reductase. It is assumed that bacteria are capable eliminate the dyes due to a variety of enzymes. Similarly, fungal enzymes have the capacity to oxidize a wide spectrum of colors (Ngo and Tischler, 2022; Selvaraj et al., 2021).

Oxidoreductive enzyme systems of bacteria, like laccase, DCIP-reductase, and azo reductase, are suggested to degrade dyes both within and outside of cells. Some microorganisms degrade azo compounds in anaerobic settings. The breakdown of the precursors may produce colorless aromatic amines (Qiu et al., 2022). The whole process cannot be finished unless these aromatic chemicals that are formed as metabolites during biodegradation are eliminated. This is significant since research has shown that these biotransformation products can be hazardous, carcinogenic, and mutagenic under certain conditions (Ngo and Tischler, 2022; Al-Tohami et al., 2022). The objective of this investigation was to optimize the degradation efficiency of *P. aeruginosa* strains against methyl red dye.

2. Material and methods

2.1. Dye and Other Reagents

Methyl red textile azo dye was obtained from a local factory. The excellent analytical grade and quality of the nutritional broth, sodium hydroxide, glucose, n-hexane, ethyl acetate, hydrochloric acid, redox mediators, and other chemical reagents were utilized for current work. Sigma Aldrich was the source of the remaining chemical reagents.

2.2. Preparation of Dye Solution

The prepared stock solution had 500 ml of double distilled water and one g of methyl red dye. A range of dye solutions having amounts 100 mg/l to 800 mg/l (100 ppm to 800 ppm) were made separately in different containers by utilizing stock solution. For the reason that azo dyes becomes unstable when sterilized with moisture and heat, the sterilization of solutions was completed by a 0.22 µm membrane filter.

2.3. Conditions of medium and culture

Mineral salt medium (MSM), comprising of 4 g K2HPO4; 4 g KH2PO4; 2 g (NH4)2SO4; 0.5 g MgSO4•7H2O; 0.01 g CaCl2; and 0.01 g FeSO4•7H2O per liter of distilled water, was the fundamental medium utilized. A total of 100 mL of MSM, 0.1% glucose, 0.4% yeast extract, and 50 mg/ml dye were added to 250 ml Erlenmeyer flasks for the majority of tests.

2.4. Type of Bacteria

The biological degradation effectiveness of *Pseudomonas aeruginosa* clinical isolates was examined using methyl red dye. The cultures mentioned above were obtained from Aligarh Muslim University, India.

2.5. The impact of dye concentration on biodegradation

In order to study the impact of dye concentration on breakdown, the selected strain was cultured in eight tubes using 15 ml of nutritious broth for a whole day. 5 ml of methyl red solution, varying in concentration from 100 mg/l to 800 mg/l (100 ppm to 800 ppm), was added to the test tubes separately once the colony had developed. The culture mixture containing the dye-degraded products was centrifuged for 600 seconds at a speed of 10,000 rpm. Filter paper of 0.2 µm pore size was used to filter the material. With the use of a colorimeter, the extracted supernatant mixture's absorbance was investigated at 430 nm. After incubation for six days, each dye's maximal max (430 nm) decolorization percentage (%) was calculated by following formula.

$$\% \text{ Decolorization} = (\text{Initial absorbance} - \text{final absorbance})/\text{Initial absorbance} \times 100. \quad (1)$$

2.6. Temperature impact on dye biodegradation

To find out if temperature influenced the breakdown of methyl red, 10 ml of solution was added to six test tubes, which were then infected with the selected culture. Each test tube was provided an extra five milliliters of the concentration of 40 ppm methyl red stock solution after the bacteria had grown. Additionally, a control solution consisting of 5 ml of methyl red and 10 ml of nourishing broth was prepared. Test tubes were incubated in the incubator at 17, 20, 23, 26, 29, 31, 34, 37, 40, 43, 46, and 49 °C. Using filter paper with a size of 0.2 µm, the deteriorated samples were filtered after being centrifuged for 10 minutes at 10,000 rpm. To determine the percentage of decolorization, calculation was made using previous formulae (1).

2.7. Glucose Effect on Dye Biodegradation

Glucose may serve as a provider of energy as well as carbon. After 24 hours of incubation, each inoculated test tube having dye solution was provided a 5 ml of the dye solution and different amounts of glucose (100–1400 mg/l). The bacteria were cultured at 37°C in test tubes using nutrient broth medium. Control solutions comprising 5 ml of dye solution and 10 ml of medium only were also prepared for all glucose concentrations. The degraded sample was examined using colorimeter after centrifugation and filtering using 0.2 µm filter paper. To determine the percentage of decolorization, calculation was made using previous formulae (1).

2.8. Effect of pH on dye biodegradation

To find out how different pH values (1, 2, 3, 4,5, 6, 7, 8, 9, 10, 11, and 12) affected the decolorization, a constant concentration of methyl red (50 mg/l) in 50 ml of MSM supplemented with 0.1% glucose and 0.4% yeast extract was studied under static circumstances and at 30 °C for 24 hours. Since bacteria are able to survive at a certain pH, sterile nutritional broth was poured to fourteen test tubes, *P. aeruginosa* was introduced into them, and the tubes were subsequently incubated at 37 °C in an incubation chamber. After 24 hours, when the culture of bacterial cells had attained its maximum size, 5 milliliters of the original solution's dye solution had been added to each test tube. As a blueprint, control solutions were also developed.1 M NaOH and 1 M HCl solution were used to modify the pH levels in the inoculation tubes and control reference solutions. The pH readings were changed using pH indicator strips (Merck KGaA Darmstadt, Germany) by introducing a little amount of base or acid using a micropipette in the appropriate tubes. Following centrifugation, filter paper with a 0.2 µm pore size was used to filter the supernatant. Using the previously described method (1) and a colorimeter, the percentage of decolorization of the supernatant removed was determined after three days.

2.9. Sodium chloride effect on dye biodegradation

Dyes usually break down under optimal salinity conditions. Due to its great ability to enhance the salinity of seawater, sodium chloride salt exerts an effect on the process of dye degradation. Test tubes containing *P. aeruginosa* culture were supplied with 5 ml of dye solution. Sodium chloride was also added to each test tube with innoculum, varying in dosage from 100 to 1400 mg/l. There was also reference solutions prepared for every concentration of sodium chloride. Using the obtained supernatant from centrifugation, the degree of decolorization was computed. More investigation is necessary, nevertheless, in order to properly mineralize the treated effluent prior to its release into the environment or its suitability for reuse.

2.10. Impact of incubation time on dye biodegradation

A big test tube was filled with 30 milliliters of nutritional broth, injected with a bacterial culture, and allowed to incubate for a whole day. The culture was developed, and then 15 milliliters of dye solution were added. The proportion of dye degradation was monitored from one day to fifteen days. Also prepared as a point of comparison was a control solution containing 10 ml of medium and 5 ml of dye. The colorimeter was used to assess the % deterioration rate up to a period of 15 days.

2.11. Effect of heavy metal ions

The effects of these metals on decolorization activity were investigated by experiments using doses of 1 and 5 mM. Among these metal ions were Hg ($HgCl_2$), Zn ($ZnSO_4$), Mg ($MnCl_2$), Co ($CoCl_2$), and Mg ($MgCl_2$). Flasks containing the dye were added after the cell suspension was incubated for 15 minutes with metal ions supplied from stock solutions. Decolorization was seen throughout a variety of time intervals at 30 °C.

2.12. Statistical analyses

Minitab software (Version 17) was used to do statistical analysis and graphics for multifactorial design. Graph Pad Prism 5 software was utilized for the majority of statistical studies, including Dunnett's multiple comparisons post-test at P-value < 0.05 and one way ANOVA.

3. Results

3.1. Effect of dye concentrations

This study found that as the dye concentration increased from 100 mg/l to 800 mg/l, *P. aeruginosa* capacity to remove color decreased from 125 mg/l to 800 mg/l. It was highest at 125 mg/l. It was found that when dye concentration increased, P. aeruginosa degradation efficiency dropped, as shown in Figure 1. At first doses, *P. aeruginosa* has a great capacity to degrade azo dyes, according to Cui et al. (2014). However, that potential was lessened by the high dye concentration, which caused a limited rate of bacterial growth.

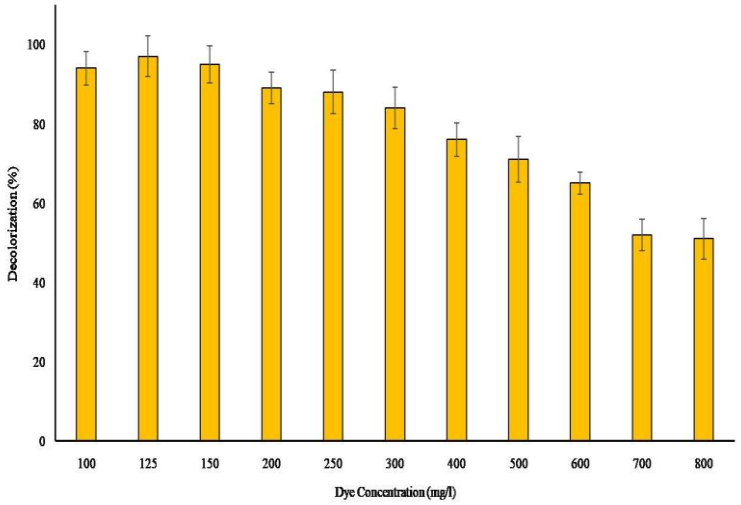

Figure 1- Effect of dye concentration on dye's decolorization.

3.2. Impact of pH on dye biodegradation

pH is a critical element that determines both the capacity for bacterial breakdown and the activity of enzymes. The effects of pH on the breakdown of methyl red are shown in Figure 2. There was an increase in degradation as the pH shifted from the acidic to the alkaline range. The results indicate that bacterial growth and enzymatic activity were affected by severe alkaline and acidic environments, as seen by the decolorization rate rising (70.35%) at pH 9 and decreasing at lower pH values (Figure 2). To decolorize dyes, alkaline conditions with pH values between 6 and 10 are typically utilized (Saratale et al., 2011).

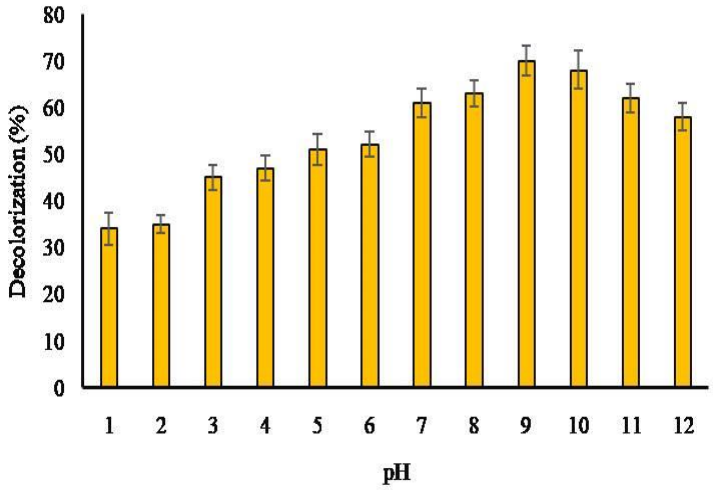

Figure 2- Effect of pH on dye's decolorization

3.3. Impact of temperature on biodegradation of dye

Temperature influences the process of biodegradation activity of bacteria. The impact of temperature on the degradation of dye can be observed in Figure 3. The impact temperature has on the growth of bacterial cells decreases the capacity of biological degradation by microorganisms. The decolorization ability of *P. aeruginosa* decreases above or below 37 °C due to the sluggish development of culture, as shown by the observation of the maximum decolorization at this temperature. High temperatures cause bacterial enzymes to go dormant, which dramatically slows down the rate at which bacteria decolorize (Anjaneya et al., 2011). The optimal growing temperature for the degradation of reactive azo dyes is found to vary from 35 to 45 °C until the maximal dye degradation is obtained (Pearce et al., 2003).

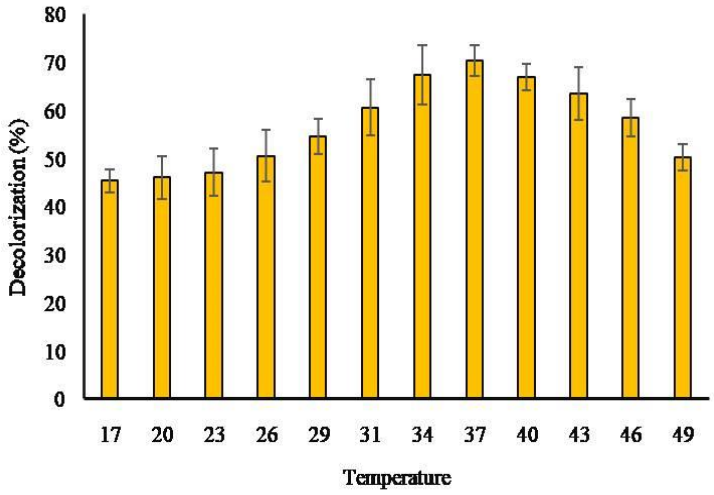

Figure 3- Effect of temperature on % decolorization of dye

3.4. Impact of Glucose Concentration on Dye Biodegradation

Since glucose is their main source of energy and a carbon source, glucose is very significant for bacteria to exist. Certain colors are complicated by nature and difficult to break down in the context of bacteria's aptitude for degradation. According to Ikram et al. (2022), this means that extra glucose must be supplied from somewhere else. An increase in the concentration of glucose may possibly affect the rate of decolorization. But, beyond a certain point, a decline could occur in decolorization capacity of bacteria, because the bacterial metabolic pathway can no longer metabolize the sugar further (Bheemaraddi et al., 2014). Figure 4 illustrates the effect of glucose on the dye's degradation. In the present research after adding 1400.0 mg/l of glucose, a substantial degradation rate (71.08%) was observed.

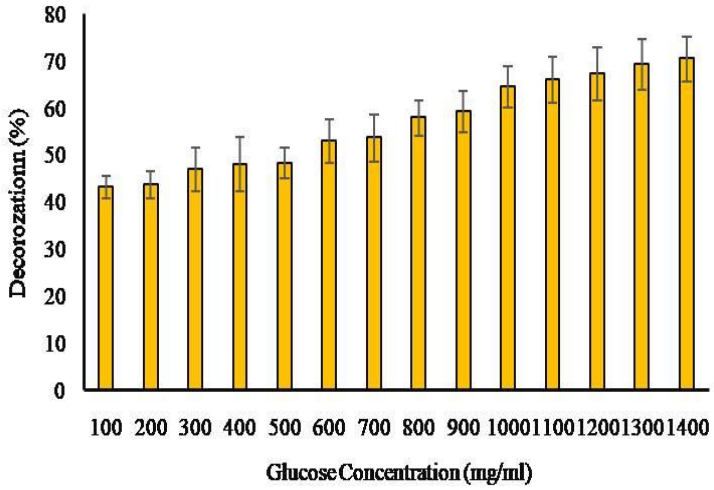

Figure 4- Effect of glucose concentration on % decolorization of dye

3.5. Impact of Urea concentration on dye biodegradation

To break down the dye, bacteria require a lot of urea since they use it as a source of nitrogen. The effect of urea concentration on *P. aeruginosa* degree of methyl red disintegration capability is shown in Figure 5. A notable rate of degradation (66.27%) was observed for the selected dye at 1000 mg/l. As the concentration of urea increased, the breakdown activity reduced because of induced toxicity. The increasing urea level (1400 mg/l) and urea toxicity caused the percentage breakdown activity to drop to 59.59 percent at even greater concentrations.

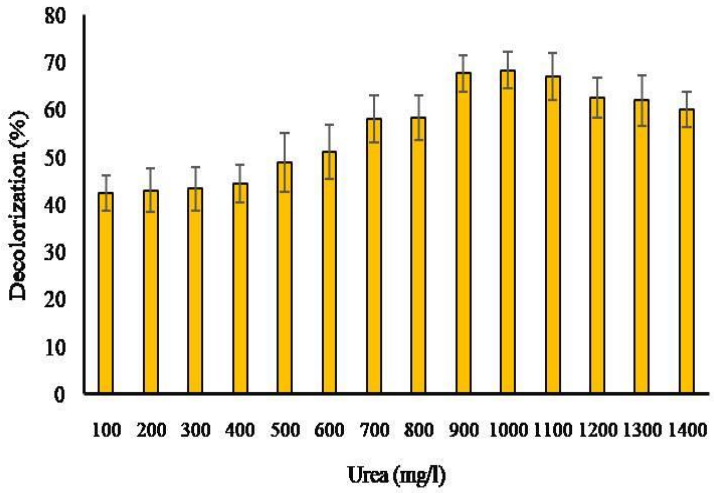

Figure 5- Effect of urea concentration on % decolorization of dye

3.6. Impact of Incubation time on dye biodegradation

The degradative activity of bacteria is also influenced by time. The effect of time on *P. aeruginosa*'s capacity to degrade methyl red is shown in Figure 6. Every day during the first six days, the dye's deterioration was observed. Since there was no discernible increase after 6 days, the percentage degradation was evaluated every 3 days up to 21 days. It had reached it's a significant degree of decolorization after three days of incubation. After these three days, the rate of decline was not so great. The most likely explanation for this is because the stationery and mortality phase is almost upon us.

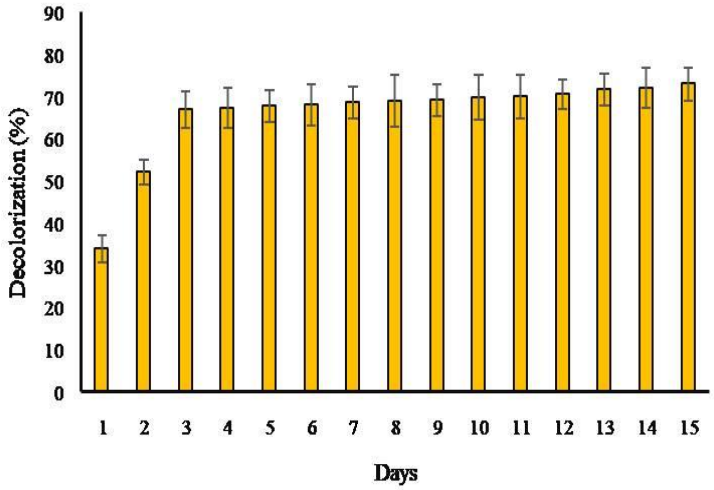

Figure 6. Incubation time (days) impact on % degradation of dye

3.7. Effect of Salinity on dye biodegradation

The impact of sodium chloride concentration upon the methyl red degradation by a selected type of bacteria has been shown in Figure 7. The ability of microorganisms to degrade dye is found to decrease with increasing concentration of saline. At a salt concentration of 700 mg/l, *P. aeruginosa* was shown to breakdown methyl red at the highest percentage. In high salinity environments, bacteria experience plasmolysis, which limits their growth and, consequently, their ability to break down materials (Ikram et al., 2022).

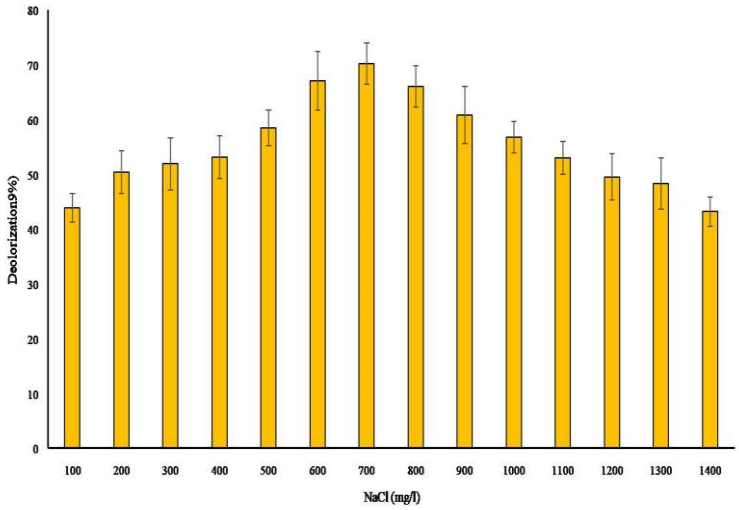

Figure 7- Effect of NaCl concentration on % decolorization of dye

3.8. Impact of presence of heavy metal ions on dye biodegradation

Textile effluents are especially dangerous since heavy metals are commonly found in them. Specific metal ions may be advantageous for bacteria, but other ones can be hazardous. Heavy metals directly impact proteins or enzymes because they form bonds with protein molecules in order to form complexes (Balali-Mood et al., 2021). So, we looked at how different metal ions at concentrations of 1 and 5 mM affected P. aeruginosa ability to decolorize. Figure 8 shows that only Mn had a marginally inducing effect on decolorization performance (94%). Zinc was the element that inhibited the most at both concentrations among the ones that were investigated. Co and Hg also had a significant negative impact at 5 mM values. Mg did not exhibit a significant impact on decolorization of methyl red.

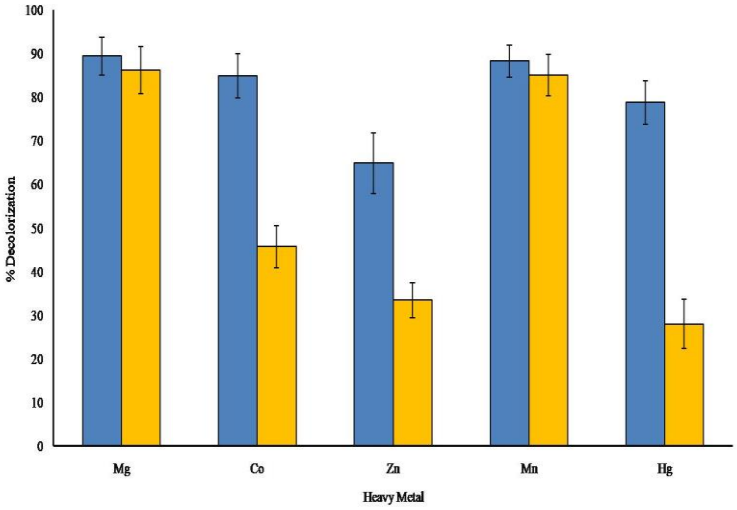

Figure 8. Effect of presence of metal ions on decolorization of methyl red

4. Discussion

The treatment of effluent from textile industry is very much compulsory due to the hazardous nature of it. In addition to its toxic effects on human beings, synthetic dyes have the capacity to cause more serious adverse effects on ecosystems. These synthetic dyes can hinder the capacity of plant to do photosynthesis. These dyes may alter and decrease light and oxygen solubility in water. Besides, azo dyes can inhibit the growth of plants. The quality of the water may be impacted optically through these dyes. Due to their naturally persistent chemical structure, which promotes a range of unfavorable interactions, these dyes have unfavorable effects (Gohatre, 2016).

The effluents having dyes diminishes a great deal of beneficial uses, such land irrigation and water potability. For this reason, artificial colors are not only detrimental to the growth and development of plants but also unappealing to the eye when present in water sources. The textile industry uses dyes that pollute aquatic environments and have the potential to be hazardous to aquatic life, which could make their way up the food chain (Sharma et al., 2021).

These colors may also inhibit the growth of seedling shoots and roots and reduce germination rates. Furthermore, the existence of aquatic species is threatened by these colors, which also disrupt the structure of aquatic ecosystems. Dye discharge without adequate treatment may affect aquatic life because it can prevent sunlight from reaching the receiving water (Ali et al., 2020). Furthermore, these waste waters can lead to a number of hazardous issues, including changes in the water's quality (color and odor) and toxicity, which can result in allergies, dermatitis, skin irritations, malignancies, and human mutations. Furthermore, due to their resistance to traditional physicochemical degradation and lack of biodegradability, 60–70% of azo dyes are poisonous, carcinogenic, and resistant to standard treatment methods. So, drainage and pollutants which included dye coming from textile and other industries need to be treated before being released into the environment (Berradi et al., 2019).

Electron transport, reactive oxygen species, and oxidative stress have been suggested to contribute to the toxicity of dyes (Kovacic and Somanathan, 2014). Oxidative stress occurs due to imabalance of production and elimination of reactive oxygen species (Rahmani et al., 2023; Rahmani et al., 2022; Anwar and Younus, 2018). Reactive oxygen species are extremely reactive chemical species that contain oxygen and are constantly produced by the body as a result of cell metabolism (Anwar et al., 2023; Anwar et al., 2022). Antioxidants often prevent or reduce toxicity (Kovacic and Somanathan, 2014). Alcohol dehydrogenase is an important antioxidant as a chief defense against oxidative stress (Haque et al., 2012). The synthetic coloring agents including dyes are used extensively across various sectors, like paper printing industry, rubber industry, groceries, and beauty products sectors, along with infrastructure (Mohan and Sharma, 2022). Azo dyes are a class of synthetic dyes which are commonly used as one of the essential components in the textile manufacturing industry. Azo dyes have been previously shown to cause hepatotoxicity (Hussain et al., 2022). Liver is an important organ of the body (Almatroodi et al., 2020; Yahia and Anwar, 2021). So, hepatotoxicity caused by azo dyes can be very threatening for life.

The remarkable effectiveness of biological enzyme systems in the breakdown and decolorization of dyes has been already reported. They can be used in natural environments and are also much less expensive (Yaseen and Scholz, 2019).Textile dye biodegradation primarily aims to remove color, but it also transforms

hazardous textile dyes into less harmful forms that can be released into the environment without risk. Various bacterial strains have reportedly been shown to be capable of decolorizing the dyes. Since every dye has a particular structure and level of complexity, the amounts of different dyes that are destained by different bacterial strains could be varied (Kamal et al., 2022).

When exposed to azo dyes in greater quantities, microbial cells have been shown to be ineffective. These results indicate that greater concentrations of dyes could inhibit population progression through several strategies like deposition of dye and their ingredients on bacterial cells leading to slow down of microbial progression and their direct impact upon enzymes production. The aforementioned processes have the potential to impact metabolic pathways and result in hazardous secondary metabolite shock loading rates during the initial hours of the response (Ngo and Tischler, 2022).

Because of plasmolysis or the death of growing cells, a large number of bacterial species are susceptible to the high salt concentration found in the wastewater containing saline azo dye. Certain kinds of bacteria, however, have developed specific physiological and morphological features that allow them to thrive in highly salinized environments (Trivedi et al., 2022). Elevated NaCl levels may encourage lyses of biomass, which would supply sufficient carbon from organic matter to enable efficient denitrification. Physical and chemical factors that affect the isolate's capacity to decolorize textile colors include temperature, pH, dye concentration, and carbon availability (Ikram et al., 2022).

This experiment was therefore carried out to ascertain whether our developed procedure could be maintained under conditions of excessive salinity. The ideal pH and temperature for *P. aeruginosa* to be able to properly neutralize dye methyl red in the culture medium has been determined to be between 9.0 and 37°C, respectively, throughout the current research. According to current research, pH 9.0 produced the most decolorization (70.0%).Raising the temperature had a beneficial impact on the isolate's growth and the decolorization of methyl red, which peaked at 37°C (70.39%). The observed increases in growth and enzyme activity with temperature may be the cause of these results (Ikram et al., 2022).

Further elevation in temperature was proved to be highly restrictive for subsequent decolorization of methyl red. At a dye concentration of 125 mg/l, the isolate discolored methyl red to the greatest extent (97.18%). The proportion of decolorization decreased when the dye concentration was increased further. This could have been a consequence due to the dye's toxic effects, which impairs processes related to metabolism (Kamal et al., 2022). Effluents from textiles can be especially hazardous due to the fact that they contain contaminants such as heavy metals. Our study shows that each of the metal ions does not show adverse towards bacteria and a few of these metals may even be favorable for their growths. This study shows that heavy metals also have a negative effect on the percentage of decolorization, even at 5 mM concentration. However, heavy metals can also alter the activities of enzymes, significantly restrict growth, prevent reproduction, and other impacts on bacterial cells (Aljerf and AlMasri, 2018). The presence of heavy metals may lessen the efficiency of discoloration (Velusamy et al., 2021). Our study confirms the conclusions provided by previous researchers regarding the efficacy of *P. aeruginosa* against azo dye and provides the concept of optimum conditions for significant decolorization of effluent having azo dye as a contaminant.

5. Conclusion

In many parts of the world today, finding clean drinking water has grown difficult due to multiple causes including population growth, protracted droughts, contamination of the soil and water, and other issues. Nowadays, when water is scarce, effective water treatment is essential for surviving and promoting health and economic development. Establishing and implementing better water treatment methods that work better and require less money, is urgently essential. Our data confirm the procedure involving *P. aeruginosa* works well across a wide range of salinity and pH, which makes it appropriate for practical uses. In addition, it suggests that *P. aeruginosa* could be demonstrated to have the potential to decolorize methyl red and its derivatives as well as similar compounds. Further, the current study has unlocked the potential of *P. aeruginosa* in the purification of waters having methyl red as a pollutant and has shown them to be effective. This plan may provide a more practical and economical approach to bioremediation.

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