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Decolorization and detoxification of synthetic azo dye wastewater by *Enterobacter hormaechei* MW584986 isolated from textile wastewater

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Abstract

Biotreatment of textile wastewater is a viable option and a promising strategy for its detoxification before its discharge into the environment. This study evaluated the ability of *Enterobacter hormaechei* MW584986 isolated from textile wastewater to decolorize (degrade) trypan blue. It also identified and optimized conditions affecting both color removal and degradation processes of trypan blue using Central Composite Design (CCD) of Response Surface Methodology (RSM). Subsequently, Micro-toxicity assay was used to evaluate the detoxification of the dye by *Enterobacter hormaechei* MW584986, while generated metabolites were ascertained based on gas chromatography - mass spectometric analysis. *Enterobacter hormaechei* MW584986 effectively decolorized trypan blue synthetic dye wastewater within 24 h of incubation at 77.5% from the initial trypan blue concentration of 100 mg L^{-1}. Optimal conditions for maximum decolorization efficiency were determined to be pH 7.0, temperature 35°C, salinity 5–7 g L^{-1} and glucose concentration of 10 g L^{-1}. Micro-toxicity test showed significant growth rate of *Chlorella vulgaris* in treated broth relative to the untreated sample at same concentration, indicating the breakdown of the compound into less toxic substances. GC-MS analyses of the decolorized broth showed degradation of azo dye into simpler substances by *Enterobacter hormaechei* MW584986. This study thus reports an efficient, cheap and environmentally safe textile wastewater treatment strategy.

Keywords: Biodegradation; trypan blue dye; bacteria; RSM; GC-MS; *Chlorella vulgaris*; Azo dye; wastewater; decolorization; *Enterobacter hormaechei*; detoxification

Introduction

The discharge of untreated wastewater into the environment by the textile industry is a common practice, particularly in developing nations where regulations are lax and not practiced (Garg et al. 2020). According to the Organization for Economic Co-operation and Development (OECD 2017), the massive volumes of textile wastewater released pose a significant environmental threat to various ecosystems. These wastewaters contain a myriad of pollutants, including more than 100,000 different synthetic dyes such as anthraquinone, triarylmethane, indigoid, heavy metals, and notably, azo dyes, which are the most prevalent and make up nearly 70% of all industrial dyes (Abd El-Rahim et al. 2021; Chen et al. 2021; Liu et al. 2021; Thangaraj et al. 2021).

Azo dyes pose significant challenges in wastewater treatment due to their inherent resistance, attributed to the highly stable azo bonds (N = N) and aromatic rings within their structure. Moreover, they are known to be genotoxic and carcinogenic, presenting potential risks to both human health and the ecological environment (Chen et al. 2021; Liu et al. 2021; Thangaraj et al. 2021).

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Treating azo dye-laden wastewater is particularly difficult due to its high pH and salinity, compounded by the diverse chemical structures and recalcitrant nature of textile dyes.

Therefore, selecting an appropriate treatment method for dye wastewater is paramount (Al-Tohamy et al. 2023), and efforts to establish effective treatment methods for azo dye wastewater have garnered significant attention in various studies (Abd El-Rahim et al. 2021; Chen et al. 2021; Liu et al. 2021). While physical and chemical technologies are viable in the short term, they tend to be costly and can lead to secondary pollution (Liu et al. 2021). However, microbial degradation of azo dyes presents a sustainable solution and is considered a promising strategy for breaking down these stubborn substances.

Bacteria, in particular, have been extensively utilized for treating azo dye wastewaters due to their rapid reproduction, intracellular production of various redox enzymes, fast decolorization and degradation rates, and strong adaptability to harsh environmental conditions (Garg et al. 2020; Abd El-Rahim et al. 2021; Chen et al. 2021; Liu et al. 2021; Thangaraj et al. 2021). For instance, in a study on azo dyes degradation, the laccase enzyme produced by *Bacillus pumilus* ZBI exhibited significant decolorization percentages for Congo red, Crystal violet, and Reactive blue 4 (Liu et al. 2021). Additionally, Chen et al. (2021) demonstrated the detoxification of azo dye Direct Black G by the thermophilic *Anoxybacillus* sp. PDR2. Interestingly, single bacterial isolates demonstrated superior degradation compared to their consortium counterparts (Abd El-Rahim et al. 2021). *Enterobacter hormaechei* is a Gram-negative bacterium characterized by rod-shaped morphology with elongated cells, exhibiting facultative anaerobic growth with rapid proliferation in a circular configuration. Belonging to the order Enterobacterales and phylum Proteobacteria (Thangaraj et al. 2021), it thrives as an autochthonous species in polluted environments, possessing notable degradative capabilities. This bacterium has been frequently isolated from diverse sources such as activated sludge, oil-based mud, and textile mill effluent (Aissaoui et al. 2016; Thangaraj et al. 2021; El-liethy et al. 2022). *Enterobacter hormaechei* SKB 16 has demonstrated remarkable decolorization efficiency, achieving an 80% reduction in Reactive Yellow 145 and Reactive Red 180 within 72 hours during both shake flask and static remediation processes (Thangaraj et al. 2021).

This investigation assessed *Enterobacter hormaechei* MW584986 ability to decolorize (degrade) trypan blue in textile wastewater. Using the Central Composite Design (CCD) of Response Surface Methodology (RSM), the study identified and optimized conditions affecting both color removal and degradation processes of trypan blue. Following this, the generated metabolites were analyzed, and a micro-toxicity assay was performed to evaluate the detoxification of the dye by *Enterobacter hormaechei* MW584986.

Materials and methods

Dye and chemicals

The trypan blue dye, with the chemical index number 23850, utilized in this study, was procured from Aldrich Chemical Co, USA, and is commonly employed by local dyers. It possesses high purity, being of the best analytical grade, and was utilized without prior purification. All chemicals employed in the study were of analytical grade. The chemical structure of trypan blue is depicted in Fig 1.

Isolation and identification of bacteria

The textile effluent utilized for bacterial isolation was generously provided by Sunflag Nigeria Limited and Kofar Mata Dye Pits. Enrichment of the textile effluent samples was conducted by inoculating 10 mL of the samples into 90 mL of mineral salt broth enhanced with 0.01% of trypan blue dye, contained within 250 mL Erlenmeyer flasks, following the method described by Thakur *et al.* (2018). Serial dilutions of the enriched culture of the textile effluent were performed up to dilution 10^-3. This process involved adding 1 mL of the enriched broth culture of the effluent into 9 mL of

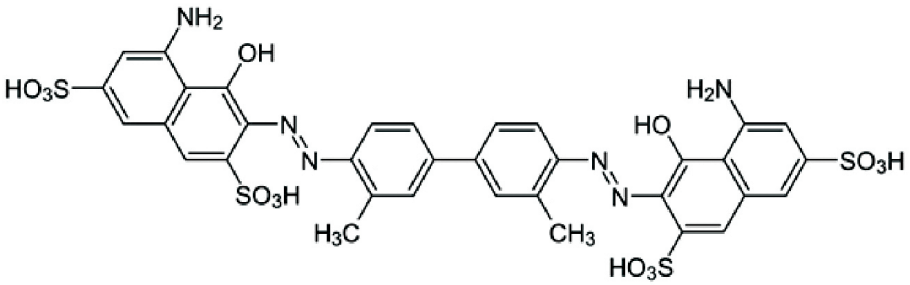

Figure 1. Chemical structure of Trypan Blue (C.I. 23850)

0.1% peptone water and thorough mixing with a vortex mixer. From dilution 10^-2, 1 mL was transferred into another fresh and sterile diluent to create dilution 10^-3. Subsequently, mineral salt agar medium enhanced with 0.01% of trypan blue dye was inoculated with 0.1 mL of each suspension, spread using a glass spreader, and then incubated at 37°C for up to 72 hours. Following incubation, the zone of clearance around distinct colonies was regarded as evidence of decolorization, and the organisms exhibiting the highest zone of clearance were identified. Bacterial identification was based on morphological, physiological, and biochemical tests, as well as 16S rRNA marker-based molecular approaches. All biochemical tests were conducted using designated methods, and microbial cultures were differentiated using rDNA restriction analysis of the 16S RNA genes sequencing and Polymerase Chain Reaction (PCR) with appropriate primers and method procedures rigorously followed (Mohana et al. 2007). The obtained sequences were submitted to NCBI, and accession no. *Enterobacter hormaechei* MW584986 was obtained from the Gene Bank. The bacteria were routinely maintained at 37°C in Bushnell and Haas broth, and subsequently, their ability was exploited for the decolorization of the diazo dye trypan blue.

Decolorization studies

Decolorization experiments were conducted using nutrient broth (95 mL) supplemented with 0.1 g of the azo dye (trypan blue) and inoculated with 5 mL of *Enterobacter hormaechei* MW584986, with the inoculum adjusted to 0.5 McFarland standard solution. Following incubation for 24 h, each broth culture was centrifuged at 12,000 rpm for 15 minutes to determine the percentage of decolorization as indicated below. For assessing the growth of *Enterobacter hormaechei* MW584986, the broth culture was not subjected to centrifugation. The absorbance of both the supernatant and the decolorized broth was measured using a UV-visible light spectrophotometer (LaMotte Smart) at 580 nm, while bacterial growth was monitored at 600 nm. Uninoculated dye broth served as the control (blank) in the study.

$$\text{Decolorization \%} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$$

Optimization of culture conditions using central composite design for dye decolorization. Conditioned culture for dye decolorization

Modifying the culture conditions to improve the bacterial decolorization process involved adjusting key factors such as varying pH (4 -10), temperature (15°C - 45°C), sodium chloride levels (0.5-10 g L^{-1}), and glucose concentrations (0.1 – 10 g L^{-1}). These parameters play a crucial role in influencing the dye decolorization process, as highlighted by previous studies (Chen et al. 2003; Khehra et al. 2005). Therefore, a detailed examination of these variables was imperative to understand their impact on the decolorization process.

Effect of cultural conditions on biodecolorization

Effect of pH on dye decolorizations

The influence of pH on dye decolorization by bacterial isolates was investigated using varying pH levels (4, 5, 6, 7, 8, 9, and 10). Multiple sets of Erlenmeyer flasks, each containing 20 mL of mineral salt broth with 0.1 g L^{-1} dye, were prepared and inoculated with 10 mL of bacterial isolates for 24 h. Subsequently, the degree of decolorization in each flask was evaluated by centrifuging the broth samples at 12,000 rpm for 15 mins, followed by analysis of the supernatant using a UV-visible light spectrophotometer (Chen et al. 2002).

Effect of temperature on dye decolorizations

A volume of 20 mL of mineral salt broth containing 0.1 g L^{-1} of dye was inoculated with 10 mL of bacterial isolates and incubated at various temperatures (15°C, 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C) for 24 h. Subsequently, the decolorization efficiency in each flask was evaluated by centrifuging the broth samples at 12,000 rpm for 15 mins, followed by analyzing the supernatant using a UV-visible light spectrophotometer (Chen et al. 2002).

Effect of salinity on dye decolorization

Mineral salts broth containing various concentrations of sodium chloride (ranging from 0.5 to 10 g L^{-1}) and dye concentration of 0.1 g L^{-1} were employed. This medium was then inoculated with 10 mL of a bacterial isolate and incubated at 35°C for 24 h. Subsequently, the decolorization efficiency in each flask was evaluated by centrifuging the broth cultures at 12,000 rpm for 15 mins, followed by spectrophotometric analysis of the supernatant using a UV-visible light spectrophotometer (Chen et al. 2002).

Effect of various glucose concentrations on dye decolorization

Various concentrations of glucose, ranging from 0.1 g L^{-1} to 10 g L^{-1}, were incorporated into mineral salt broth with 0.1 g L^{-1} of dye. Each Erlenmeyer flask received 20 mL of this solution, which was then sterilized. Subsequently, 10 mL of an 18-hour-old culture was added and the flasks were incubated at 37°C for 24 h. Control tubes without inoculum were maintained with consistent dye concentrations across all tubes. Following incubation, the tubes were centrifuged at

12,000 rpm for 15 mins, and the resulting supernatants were analyzed spectrophotometrically (Chen et al. 2002). This procedure was repeated for all test tubes containing various sugars at different concentrations.

Central composite design (CCD) of the response surface methodology (RSM)

The response surface methodology (RSM) utilized the central composite design in Design Expert-13 to enhance the decolorization of dyes. RSM is a method employed to determine the optimal conditions for a system with multiple variables, assessing the combined impact of selected variables. In this research, RSM was applied to explore the interactions among operational factors like pH, temperature, glucose concentration, and salinity using the central composite design.

The study involved evaluating the percentage of decolorization and microbial growth at 580 nm and 600 nm, respectively, reflecting the collective influence of four variables within specified ranges: pH (5–8), temperature (25°C - 45°C), sodium chloride concentrations (0-10 g L^{-1}) and glucose concentrations (0.1-10 g L^{-1}), across 27 experimental runs (Table 1). The central composite design incorporated four independent variables as factors (Table 2) and the percentage of decolorization and microbial growth as dependent variables, with actual experimental results compared to the predicted values generated by Design Expert 13. All the experiments were performed in triplicate and the results were expressed in terms of percentage decolorization using the dependent variable calculation formula (Uppala et al. 2015) as stated below.

$$\text{Decolorization \%} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$$

Software and data analysis

The outcomes derived from the experimental design were assessed and interpreted utilizing the Response Surface Methodology (Design Expert-13) statistical software.

Table 1. Composition of various experiments of the CCD for independent variables and responses (actual and predicted) used to determine the extent of decolorization of Trypan blue by *Enterobacter hormaechei* MW584986

Run	A: pH	B: Temperature (°C)	C: Glucose Concentration (g)	D: Salinity (g)	% Decolorization		Microbial growth (600nm)	
					Actual	Predicted	Actual	Predicted
1	9	30	12	7	41.02	43.71	0.86	1.03
2	7	30	8	3	50.86	51.15	1.85	1.72
3	8	35	10	9	48.23	42.16	0.57	0.66
4	8	35	10	5	64.14	62.50	2.01	1.57
5	8	35	10	5	65.56	62.50	1.41	1.57
6	10	35	10	5	51.02	46.75	1.21	1.07
7	9	40	8	7	29.25	36.70	0.48	0.51
8	7	30	12	7	60.12	63.71	1.89	1.75
9	7	40	8	7	58.25	52.44	1.52	1.23
10	7	40	12	7	52.65	57.23	1.23	1.22
11	7	30	8	7	47.89	57.75	1.35	1.74
12	8	25	10	5	52.21	57.23	1.94	1.58
13	8	35	14	5	59.13	52.22	1.02	0.90
14	7	30	12	3	44.78	45.33	0.87	1.15
15	9	30	12	3	34.02	42.06	0.78	0.95
16	9	40	12	7	37.23	39.35	0.79	0.80
17	7	40	8	3	44.89	50.20	1.25	1.39
18	8	35	10	1	42.81	38.46	1.02	0.74
19	6	35	10	5	71.73	65.58	2.05	2.00
20	8	35	6	5	58.89	55.38	1.25	1.18
21	9	40	8	3	52.54	51.37	1.15	1.17
22	7	40	12	3	38.89	43.22	0.78	0.81
23	9	30	8	3	46.78	50.20	0.89	1.21
24	9	30	8	7	41.81	39.89	0.87	0.72
25	9	40	12	3	44.12	42.26	0.98	0.90
26	8	35	10	5	57.81	62.50	1.29	1.57
27	8	45	10	5	41.02	38.75	0.86	1.03

Monitoring of the decolorized metabolites using GC-MS

The analysis of chemical transformations in the azo dye trypan blue during decolorization was conducted using gas chromatography - mass spectrophotometry. A portion of the decolorized dye was transferred to Erlenmeyer flasks, where 15 mL of methanol was added, vigorously mixed, and left to stand for 24 h. The resulting extract was filtered through No. 1 Whatman filter paper, collected in a 100 mL conical flask, and utilized for GC-MS analysis. A 1 µL aliquot of the methanol extract was injected into the GC-MS instrument, with the oven temperature set at 60°C for 2 mins, ramped up to 300°C at a rate of 10°C per min, and held at 300°C for 6 mins. The mass detector parameters included a transfer line temperature of 240°C, an ion source temperature of 240°C, electron ionization at 70eV, a scan interval of 0.1 sec, a scan time of 0.2 sec, and a scan range of 50 to 600 Daltons. The GC-MS spectrum was analyzed using Turbo Massver 5.4.2 software and compared against the National Institute of Standards and Technology-2008 (NIST-2008) standard library database.

Microalgae assay studies

The toxicity evaluation of the decolorized synthetic dye wastewater treated with *Enterobacter hormaechei* MW584986 and untreated synthetic dye wastewater containing trypan blue was conducted using *Chlorella vulgaris* algae concentration test. Three Erlenmeyer flasks containing (a) treated dye wastewater (b) untreated dye waste water and (3) a blank medium (without azo dye added) were prepared and labeled accordingly. Subsequently, bloomed *Chlorella vulgaris* was inoculated into each flask and incubated for 12 days at 28°C. At 24 h regular intervals, samples were withdrawn, and the optical density was measured at 600 nm using a UV-visible spectrophotometer.

Table 2. Range and levels of experimental variables for decolorization by *Enterobacter hormaechei* MW584986 using Trypan blue

Factors	Levels				
	-α	-1	0	+1	+α
pH	6	7	8	9	10
Temperature (°C)	25	30	35	40	45
Glucose concentration	6	8	10	12	14
Salinity	1	3	5	7	9

Results and discussion

Decolorization performance by the isolated bacterial strain

While numerous bacterial strains exhibit the ability to degrade azo dyes, a majority of them tend to produce metabolites that are more toxic than the parent compounds and pose a greater challenge for degradation. In addition, the intricate chemical composition of dye-containing wastewaters suggests that a degrader capable of tolerating the adverse conditions of the wastewater especially, with regards to the salinity, may prove to be a more efficacious means of degrading dye compounds (Al-Tohamy et al. 2022; Guo et al. 2020). Hence, a bacterial strain capable of thriving in saline media and exhibiting varying modes of attack towards azo dye molecules, may promote the efficacy of dye wastewater bio-treatment. Therefore, the degradation extent of the textile azo dye Trypan Blue was assessed through the utilization of the newly isolated halotolerant bacterial strain in this study. The nucleotide sequence obtained from the 16 S rDNA bacterial sequencing analysis has been identified as belonging to the species *Enterobacter hormachei.* The bacterial strain had its sequences deposited to GenBank (NCBI) and was assigned the accession number MW584986. This bacterial strain has various implications in the degradation of other organic compounds (Ali et al. 2022; Al-Tohamy et al. 2020; Mo et al. 2021). The decolorization of Trypan blue synthetic dye wastewater treated with the bacterial strain was evaluated and the results suggest that the bacterial strain effectively decolorized the dye within 24 hrs of incubation to an extent of 77.5% from the initial Trypan blue concentration of 100 mg L^{-1}. The residual Trypan blue concentration after incubation for 24 hrs was 16 mg L^{-1} compared to the control that showed a residual concentration of 100 mg L^{-1}.

Effect of various parameters on dye decolorization performance

In biological treatment processes, various physicochemical parameters, such as dye concentration, pH, temperature, salinity and co-substrates, directly influence the bacterial decolorization performance of azo dyes. These parameters are crucial for making the bioremediation of wastewater containing azo dyes and high salt loads more efficient, faster, and practically applicable. Figures 2-5 depict the effect of each factor on the decolorization of Trypan Blue by the newly isolated bacterial strain.

Effect of pH

The pH of the medium is a crucial aspect in relation to the process of dye decolorization. The efficiency of dye decolorization is significantly influenced by the pH, with the ideal pH range for color removal typically falling between 6.0 and 10.0 (Kılıç et al. 2007). In this study, the decolorization of *Enterobacter hormaechei* MW584986 increased with higher pH values, with maximum decolorization at pH 7 and 8 (Figure 2). Maintaining the critical pH levels at both ends of the spectrum reduces the time required for degradation and enhances the percentage degradation rate, as noted by Mohajershojaei *et al.* (2015). At optimal pH level of 7–8, the metabolic activity of the enzyme is at its peak, which facilitates its binding to the active site of the dye surface thus, leading to a significant improvement in dye decolorization (Al-Tohamy et al. 2023). The optimal pH level exhibits a higher rate of microbial growth and color removal, whereas at highly acidic or highly alkaline pH levels, the rate of color removal tends to decrease rapidly due to the unfavorable pH conditions that limit microbial growth (Figure 2). At lower pH levels, the efficacy of decolorization decreases as dye cations cannot compete with H^+ ions. The maximum electrostatic attraction force between the negatively charged surface of biomass and the positively charged dye cations is observed at high pH levels (Al-Tohamy et al. 2023). The process of decolorization is known to be impeded by the rate-limiting phase of dye molecule transportation through the cell membrane into the cytoplasm where the azo bond is reduced (Martorell et al. 2017). The biological reduction of the azo bond may result in an increase in pH due to the formation of aromatic amine metabolites, which are more alkaline than the original azo compound (Ikram et al. 2022). The pH range of 7 to 8, identified as crucial for effective decolorization in this study, closely aligned with the performance of *Citrobacter* sp. CK3, which exhibited optimal decolorization of reactive red 180 at pH 6–7, as reported by Wang *et al.* (2009).

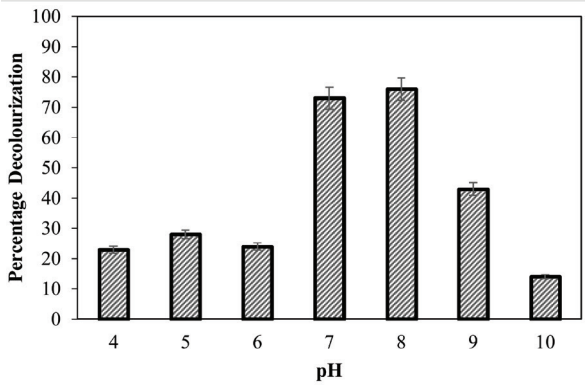

Figure 2. Effect of various pH on decolourization extent of Trypan Blue by *Enterobacter homaechei*

Effect of temperature

Temperature plays a crucial role in influencing the growth rate, survival, and metabolic activities of microorganisms. The optimal temperature for *Enterobacter hormaechei* MW584986 in dye decolorization was 35°C. According to Guan *et al.* (2020) and Yılmaz *et al.* (2019), the optimal temperature range for achieving dye decolorization is reported to be between 30 and 40°C. While 20°C supported bacterial growth, it interfered with and reduced the percentage of decolorization compared to the optimal temperature (Figure 3). Conversely, 45°C was unsuitable for microbial growth, impacting the rate of decolorization likely due to enzyme denaturation. Generally, the decolorization rate of azo dyes increases up to the optimal temperature, after which there is a slight decrease in the decolorization activity. The decline at higher temperatures can be attributed to either the degradation of cell viability or the inactivation of enzymes responsible for the catabolic breakdown of azo dyes, as reported by Wu *et al.* (2022). Nevertheless, the bacterial strain demonstrates the ability to adapt to a wide range of temperatures, showing versatility in various thermal conditions. These findings align with previous research on the degradation of 100 mg L^{-1} of Congo red at 37°C by a bacterial consortium, as reported by Holey (2015).

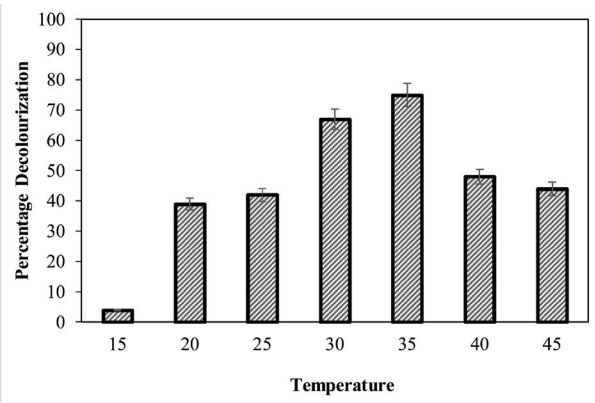

Figure 3. Effect of different temperature on decolourization extent of Trypan Blue by *Enterobacter homaechei*

Effect of salinity

Industrial salt plays a vital role in textile industries as it facilitates dye penetration into materials and binding to fibers. However, the generated hypersaline conditions in wastewater after the dyeing process often hinder microbial activity, growth, and survival, making them a significant challenge in implementing microbial wastewater remediation strategies. The high salinity levels found in dyeing wastewater pose a substantial hurdle, as many naturally occurring microorganisms

are highly sensitive to salt concentrations, as reported by Guo *et al*. (2020). In this research, the optimal salinity range required by *Enterobacter hormaechei* MW584986 was determined to be between 1–2,5 g L^{-1} of salt suggesting the halotolerance status of the bacterial strain and the halo-dependent nature of the decolorization enzymes (Figure 4). This salinity level supported the growth of the inoculated bacteria, whereas at 5 g L^{-1} and 10 g L^{-1} of salt, microbial growth was hindered, consequently affecting the rate of dye decolorization. Hypersaline environment can cause cell plasmolysis and cellular inactivity, as noted by He *et al*. (2017). High salt levels can also lead to a significant decrease in enzyme activity, potentially resulting in complete inactivation, as reported by Sarkar *et al*. (2020). Therefore, it is essential to screen and isolate halotolerant or halophilic microorganisms to improve the bioremediation process for textile effluent containing azo dyes and high salt loads.

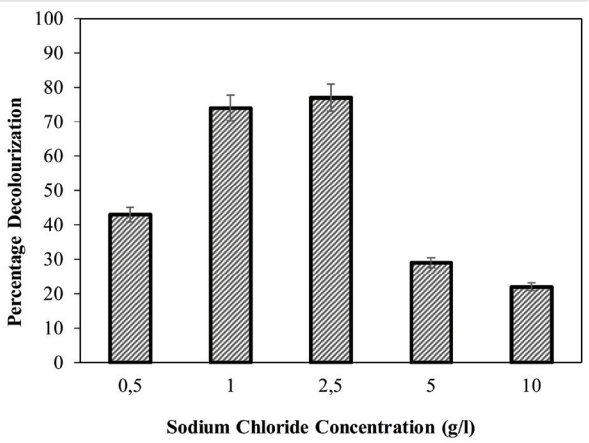

Figure 4. Effect of salinity on decolourization extent of Trypan Blue by *Enterobacter homaechei*

Effect of glucose concentration

Figure 5 illustrates the decolorization efficiency of Trypan blue in the presence of a co-substrate, glucose, by *Enterobacter hormaechei* MW584986. Since azo dyes do not serve as direct carbon sources for microbes, the inclusion of a co-substrate is essential to stimulate initial microbial growth and activity for the eventual decolorization of the azo dye. The research examined different concentrations of glucose, with the optimal range showing the most significant impact at 10 g L^{-1} (Figure 5). Previous studies have highlighted that decolorizing azo dyes with both mixed and pure cultures often require the use of complex organic sources, such as a combination of intricate organic sources and carbohydrates, as noted by Khehra *et al*. (2005). In the decolorization of azo dyes through the reduction of azo bonds, reducing equivalents derived from the catabolism of various carbon sources are transferred to the dye, facilitating its reduction and decolorization, as reported by Popli and Patel (2015). For instance, the decolorization of Reactive Violet 5 was successfully improved with glucose as a carbon co-substrate by a bacterial consortium, as noted by Moosvi *et al*. (2007). Furthermore, in various studies, the addition of carbon sources, particularly glucose concentration, has been demonstrated to enhance the percentage decolorization of microbial systems (Haug et al. 1991; Kapdan et al. 2000).

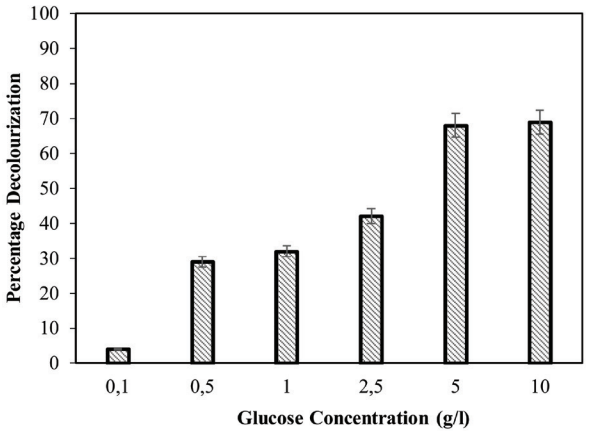

Figure 5. Effect of various glucose concentrations on decolourization extent of Trypan Blue by *Enterobacter homaechei*

Optimization of the decolorization process using the CCD

The analysis of main effects revealed that pH, temperature, salinity and glucose concentration significantly influenced the decolorization efficiency of azo dyes. Optimal decolorization was achieved at a pH of 7, temperature of 30°C to 35°C, salinity of 1–2.5 g L^{-1} and glucose concentration of 10 g L^{-1}. These findings are consistent with previous studies indicating the importance of these parameters in the decolorization process (Cao et al. 2019; Trivedi et al. 2022).

Furthermore, interaction effects between pH and temperature, as well as between temperature and salinity were observed. These interactions highlight the complex nature of the decolorization process and underscore the need to consider multiple variables simultaneously for optimal results. Similarly, the temperature's significant impact on decolorization efficiency underscores its role in driving biochemical reactions. Elevated temperatures enhance enzyme activity and substrate solubility, leading to improved decolorization. However, excessively high temperatures may induce enzyme denaturation or thermal degradation of the dye, compromising efficiency.

The interaction effects between pH and temperature, as well as between temperature and initial dye concentration, highlight the synergistic or antagonistic relationships between these variables. The pH-temperature interaction suggests that certain pH-temperature combinations may promote enzyme-substrate interactions, optimizing decolorization efficiency. Conversely, the temperature-glucose concentration interaction underscores the importance of considering co-substrate availability in temperature-dependent processes. The response surface plots and contour plots provided valuable insights into the nature of these interactions, enabling the identification of optimal operating conditions.

Model fitting and statistical analysis

The experimental data obtained from the central composite design (CCD) were fitted to a quadratic model to evaluate the relationship between the independent variables (pH, temperature, salinity and glucose concentration) and the response variable (decolorization efficiency). The adequacy of the model was assessed through analysis of variance (ANOVA). The results of the ANOVA indicated that the quadratic model was highly significant ($p < 0.05$), suggesting that it adequately represented the variability in the decolorization efficiency. The coefficient of determination (R-squared) value of 0.92 indicated that the model explained 92% of the variability and reproducibility in the response variable, further confirming its suitability for predicting decolorization efficiency.

The results of this study demonstrate the effectiveness of Response Surface Methodology (RSM) in optimizing the decolorization process of azo dyes. The optimization of azo dye decolorization using Response Surface Methodology (RSM) yielded compelling results, showcasing the nuanced effects of pH, temperature, and initial dye concentration on decolorization efficiency and their interactions (Figs 6–11). The robustness of the fitted quadratic model underscores its efficacy in capturing the complex interactions between these variables and the response. The quadratic model fitted to the experimental data adequately represented the relationship between the independent variables (pH, temperature, salinity and glucose concentration) and the response variable (decolorization efficiency), as evidenced by the significant p-value (< 0.05) obtained from the analysis of variance (ANOVA) and the high coefficient of determination (R-squared) value of 0.77. These findings suggest that the model can accurately predict decolorization efficiency based on the specified process parameters. The phenomena observed in our study can be attributed to the complex interplay between various factors influencing the decolorization process. pH, temperature, salinity and glucose concentration act as key determinants of enzyme activity, substrate solubility, and chemical reactivity, collectively influencing decolorization efficiency. The observed interaction effects further underscore the importance of considering these factors holistically to optimize the decolorization process.

Using the desirability function approach, the optimal conditions for maximum decolorization efficiency were determined to be pH 7.0, temperature 35°C, salinity 1–2.5 g L^{-1} and glucose concentration 10 g L^{-1}. Under these optimized conditions, the predicted decolorization efficiency was calculated to be 77.5%.

The optimized conditions were validated experimentally by conducting additional experiments under the determined optimal conditions. The actual decolorization efficiency achieved in the validation experiments closely matched the predicted value obtained from the model, with an average efficiency of 77.5%. This validation confirmed the robustness and reliability of the optimized model for predicting decolorization efficiency under the specified conditions.

The optimized conditions obtained in this study are in line with previous research on azo dye decolorization using various treatment methods (Amin et al. 2021). However, it is worth noting that the decolorization efficiency achieved in this study (77.5%) is notably higher compared to some conventional methods reported in the literature (Garg et al. 2020; Amin et al. 2021) Notably, studies employing single-factor optimization or univariate approaches often yield lower decolorization efficiencies. For instance, while some studies achieved high efficiencies at specific pH or temperature ranges, they often neglected interactions between variables. By incorporating RSM, our study comprehensively explored these interactions, leading to a superior outcome. This improvement can be attributed to the systematic optimization of process parameters using RSM, which allowed for the identification of optimal conditions leading to enhanced efficiency.

The optimized conditions determined in this study (pH 7.0, temperature 35°C, salinity 1–2.5 g L^{-1} and glucose concentration 10 g L^{-1}) can serve as practical guidelines for industrial-scale applications of azo dye decolorization. By adopting these optimized conditions, industries can achieve higher decolorization efficiencies while minimizing resource consumption and operational costs. Moreover, the environmentally friendly nature of the optimized process parameters aligns with sustainability goals and regulatory requirements.

Micro algae assay

The growth responses of *Chlorella vulgaris* in synthetic media at 28 °C for 21 days, as depicted in Figure 12, showcased the optical density measurements at 660 nm across varying dye concentrations. The positive control (PC) consisted of *Chlorella vulgaris* and synthetic media, while the negative control (NC) included treated

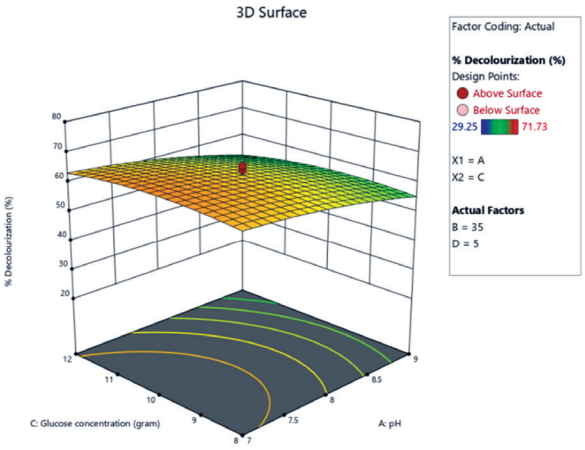

Figure 6. 3.D surface plots for the optimization of Trypan Blue decolourization by *Enterobacter hormaechei* (MW584986) as a function of pH and glucose concentration

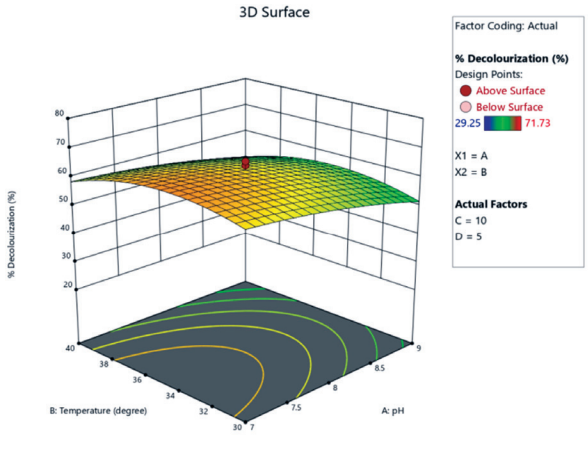

Figure 7. 3.D surface plots for the optimization of Trypan Blue decolourization by *Enterobacter hormaechei* (MW584986) as a function of pH and temperature

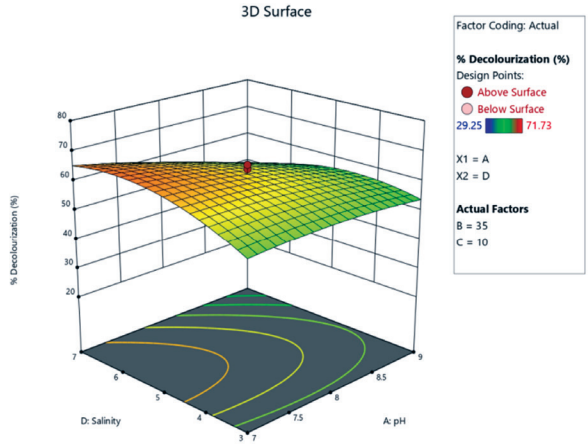

Figure 8. 3.D surface plots for the optimization of Trypan Blue decolourization treatment by *Enterobacter hormaechei* (MW584986) as a function of pH and salinity

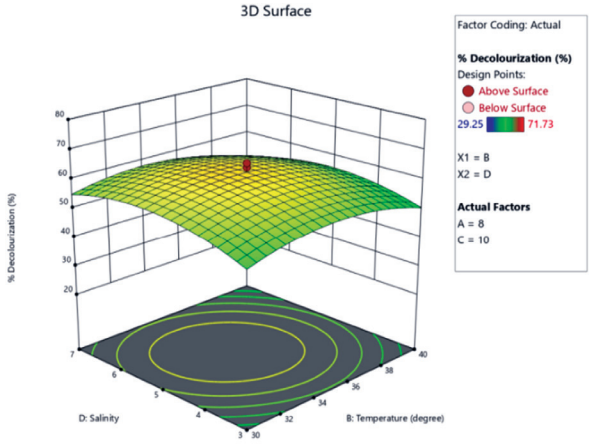

Figure 9. 3.D surface plot for the optimization of Trypan Blue decolourization by *Enterobacter hormaechei* (MW584986) as a function of temperature and salinity

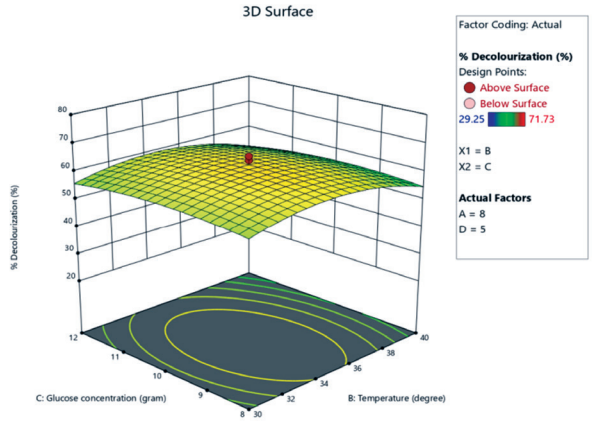

Figure 10. 3.D surface plots for optimization of Trypan Blue decolourization by *Enterobacter hormaechei* (MW584986) as a function of temperature and glucose concentration

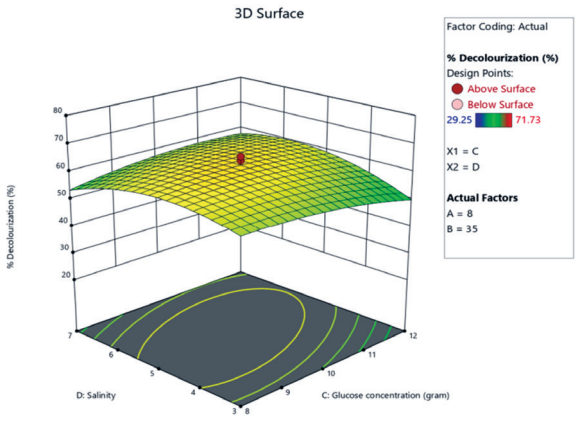

Figure 11. 3.D surface plots for the optimization of Trypan Blue decolourization by *Enterobacter hormaechei* (MW584986) as a function of glucose concentration and salinity

decolorized broth, *Chlorella vulgaris*, and synthetic media, serving as baselines for comparison at different dye masses (0.001 g L^{-1}, 0.01 g L^{-1}, 0.02 g L^{-1}, 0.03 g L^{-1}, 0.04 g L^{-1}, 0.05 g L^{-1}, 0.06 g L^{-1}, and 0.1 g L^{-1}). The chart illustrates that higher dye masses correlate with increased toxicity, leading to greater inhibition of growth rates. Notably, the treated broth exhibited a significant growth of the microalgae during incubation compared to the untreated sample, indicating the breakdown of the compound into less toxic substances. Previous studies have highlighted the carcinogenic and mutagenic nature of azo dyes and their metabolites, emphasizing the need for accurate risk assessment of treated wastewater on both aquatic animals and humans (Chen et al. 2021).

Contrastingly, biological treatment of azo dye toxic solutions proved superior to untreated solutions in promoting the growth of plant seeds (statistically significant at $P < 0.05$). Seeds watered with low-concentration of treated azo dye solutions showed growth potential comparable to those watered with distilled water, suggesting a reduction in azo dye toxicity post-treatment with *Enterobacter hormaechei*. This study underscores the potential of strain *Enterobacter hormaechei* in bioremediation of azo dye wastewater, offering insights for pollution-free treatment and practical applications in agricultural irrigation (Chen et al. 2021). Enzyme activities in plants treated with low-concentration dye metabolites closely resembled those treated with distilled water, indicating a significant reduction in the plant defence system when exposed to biologically treated azo dye solutions compared to untreated counterparts. Thus, strain *Enterobacter hormaechei* exhibits substantial promise for azo dye wastewater bioremediation. The study's findings, suggest the efficacy of azo dye treatment in reducing toxicity.

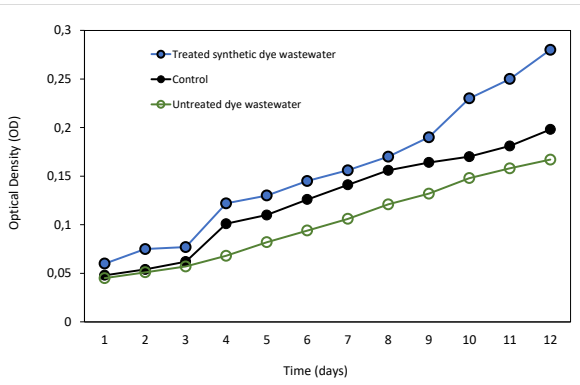

Figure 12. Growth of *Chlorella vulgaris* (measured as Optical density) in treated and untreated synthetic dye wastewater

GC-MS analysis

UV-visible spectrophotometry and GC-MS analysis were employed to assess the degradation of trypan blue following decolorization by *Enterobacter hormaechei* MW584986. The observed reduction in the maximum absorption wavelength at 580 nm indicates the cleavage of the two N=N bonds within the azo dye's chromophore, leading to its breakdown into aromatic amines and/or simpler compounds. Furthermore, a decline in both the intensity and variety of peaks in the chromatograms (Figs. 13 and 14) from 47 to 12 signifies the degradation of the dye, its impurities, and other compounds present in the medium. The untreated wastewater had a myriad of compounds (47) that were decimated to 12 metabolites with highly attenuated peaks suggesting mineralization at the end of dye decolorization. This indicates that the bacterium also possessed the ability to degrade other compounds asides the azo dye. This data serves as additional confirmation of the biotreatment of synthetic dye wastewater.

The degradation of dye structures involves various enzymes that play crucial roles in breaking down the azo bond. Azo reductase and NADH-DCIP reductase, as highlighted by Song et al. (2018), facilitate the reductive cleavage of the azo bond. Additionally, enzymes like veratryl alcohol oxidase aid in oxidation cleavage and desulphonation reactions necessary for dye degradation (Phugare et al. 2011). Tyrosinase, a tetrameric enzyme, is instrumental in the hydroxylation reaction essential for textile dye degradation (Veismoradi et al. 2019). These enzymes are active in metabolic pathways such as glycolysis, gluconeogenesis, and the tricarboxylic acid (TCA) cycle, generating reducing power that aids in azo bond reduction by reductase enzymes in bacterial cells. The initial step in azo dye biodegradation typically involves the reduction cleavage of azo bonds. Azo dyes often contain toxic components like aniline, amino-benzenesulfonic acids, benzidine, and naphthalene, necessitating energy-intensive aromatic-ring cleavage for detoxification (Al-Tohamy et al. 2023). In the case of trypan blue, the two azo bonds are expected to undergo cleavage by azo reductase, leading to the formation of various metabolites as potential intermediates. These metabolites that are mainly aromatic compounds undergo ring cleavage and further breakdown into simpler compounds. This study sheds light on the capabilities of a novel dye-degrading bacterial strain in the degradation of trypan blue and its impurities in a synthetic dye wastewater.

Conclusions

In conclusion, this study demonstrated that *Enterobacter hormaechei* MW584986 isolate from synthetic textile wastewater exhibited the ability to degrade azo dyes, such as Trypan blue. Utilizing

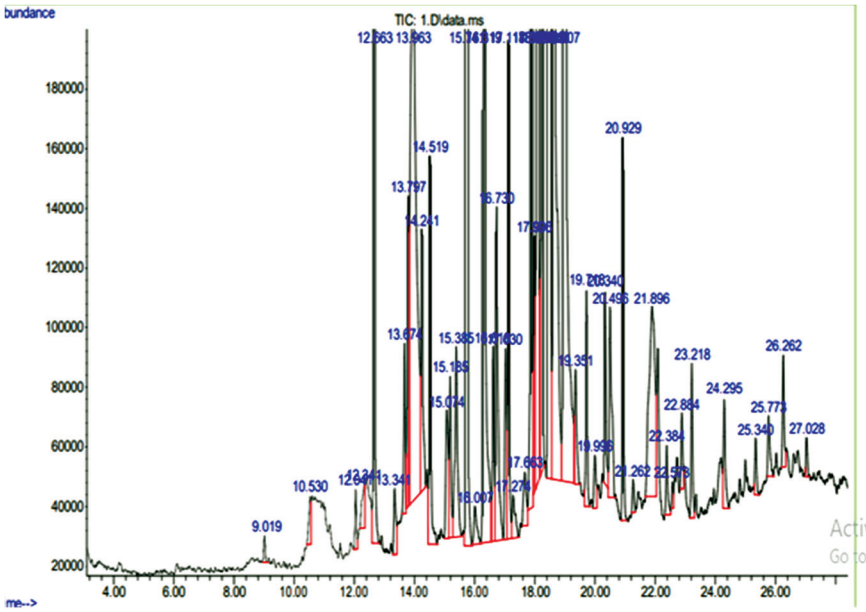

Figure 13. GC-MS chromatogram of the synthetic dye wastewater before biotreatment using *Enterobacter hormaechei*

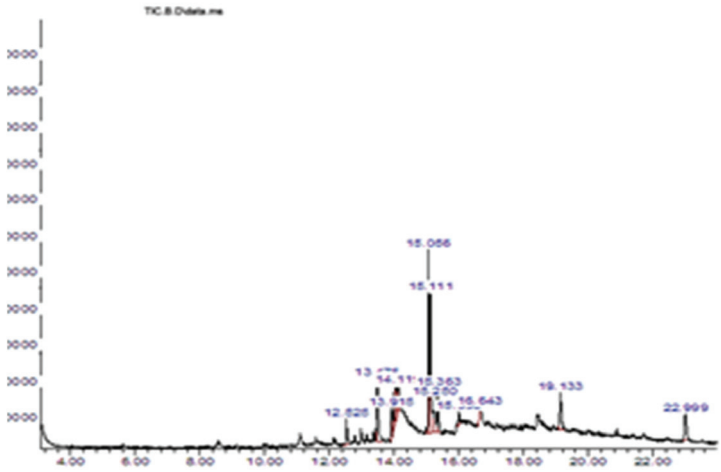

Figure 14. GC-MS chromatogram of the synthetic dye wastewater after biotreatment using *Enterobacter hormaechei*

Response Surface Methodology with a central composite design, the dye was effectively decolorized, and the resulting metabolites were analyzed using GC-MS chromatography. The efficacy of dye detoxification was confirmed through a microalgal assay following treatment with *Enterobacter hormaechei* MW584986. The results highlight the significance of pH, temperature, salinity and glucose concentration as critical factors influencing decolorization efficiency. By systematically optimizing these parameters, industries can achieve higher decolorization efficiencies while adhering to sustainability principles. Future research endeavors should focus on addressing the identified limitations and exploring innovative approaches for azo dye wastewater treatment.

Authors' Contributions

EJC was involved with data curation, methodology. and writing original draft; OCJ visualized, supervised and rote part of the manuscript, while ISO was involved with manuscript preparation, review and editing of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

"The authors declare that there are no conflicts of interest related to this article"

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