OXFORD JOURNAL
OF STUDENT
SCHOLARSHIP

Comparative Decolorization of Congo Red, Direct Black 38 and Acid Orange 7 Using Biological and Chemical Treatments

Jaisel Iyer, Hanson Shen
jaisel21@gmail.com, hanson.hh.shen@gmail.com

ABSTRACT

Azo dyes are widely used in textile manufacturing but can persist in wastewater and contribute to aquatic pollution. This study compared the apparent decolorization of three azo dyes namely Congo Red, Direct Black 38 and Acid Orange 7, under biological, natural and chemical treatment conditions over a seven day period. The treatments included yeast, *Bacillus subtilis*, white-rot fungi, pond water, aerobic and oxygen-restricted soil mixtures, hydrogen peroxide and a reverse-osmosis water control. Changes in pH and image-derived color intensity were monitored over a seven-day experimental period. Direct Black 38 showed the greatest treatment-dependent color change, particularly in yeast(65%), oxygen-restricted bacteria(60%), and Oxygen-restricted yeast(55%). Congo Red showed a more moderate and variable response, peaking at 35% color removal in aerobic yeast at 8 hours before declining to 10%, whereas Acid Orange 7 was comparatively resistant and had a maximum of 5% removal in aerobic yeast under the conditions tested. Control, hydrogen peroxide, and fungal treatments yielded 0% color removal. Changes in pH were observed in several biological treatments but did not consistently correspond with changes in color intensity. Because color removal was estimated using standardised digital photographs rather than direct chemical analysis, the findings are interpreted as evidence of apparent decolorization rather than confirmation of complete biodegradation. Degradation products and toxicity were not analyzed, so the extent of dye degradation and any change in toxicity remain undetermined. Overall, the results demonstrate that treatment effectiveness depends strongly on both the treatment system and the chemical identity of the azo dye.

INTRODUCTION

The textile industry generates substantial quantities of dye-contaminated wastewater due to its intensive use of water and synthetic colorants. Globally, the sector consumes approximately 93 billion cubic meters of water annually. Recent estimates indicate that the textile sector discharges approximately 21.5 billion m^3 of contaminated effluent annually, with wastewater from printing and dyeing processes accounting for approximately 80% of textile-sector wastewater [30]. Dyeing and finishing operations are particularly significant sources of aqueous pollution because a proportion of the dye applied may remain unfixed and subsequently enter industrial effluents. Even at relatively low concentrations, residual dyes can impart

intense color to receiving waters, reduce light penetration and interfere with the ecological functioning of aquatic environments. The complex aromatic structures that provide synthetic dyes with color stability also frequently make them resistant to conventional biological treatment and natural attenuation [1,2]. Azo dyes are among the most widely used synthetic colorants because they offer a broad range of colors, relatively low production costs, and favourable application properties. They are characterised by the presence of one or more azo linkages (–N=N–) connecting substituted aromatic structures [1].

The environmental significance of azo dyes extends beyond the visible discoloration of water. Their persistence may alter the characteristics of aquatic systems, while the organic loading associated with textile wastewater influences dissolved oxygen availability and microbial activity. More importantly, reductive cleavage of the azo bond may produce aromatic amines, some of which exhibit mutagenic, genotoxic or carcinogenic properties. For example, Direct Black 38 is a benzidine-based azo dye that can undergo microbial reduction to form benzidine and related aromatic products [3–5]. Although the subsequent degradation of these intermediates is possible under suitable microbial conditions, color removal alone does not demonstrate complete degradation or detoxification. Consequently, evaluation of azo-dye treatment requires a clear distinction between decolorization, which represents the disappearance or alteration of the visible chromophore, and biodegradation, which requires evidence that the original dye molecule has been chemically transformed [3–5].

A range of physicochemical methods have been developed for the treatment of dye-containing wastewater, including coagulation, adsorption, membrane separation, oxidation and photocatalytic processes. Some separation-based processes transfer dyes from the liquid phase to another waste stream rather than destroying them, whereas oxidative treatments may require considerable chemical or energy input. Nevertheless, unactivated hydrogen peroxide may exhibit limited effectiveness against stable azo dyes, and its performance can change substantially when combined with ultraviolet irradiation, heat, catalysts, or Fenton-type reactions [6]. Including hydrogen peroxide as a chemical-treatment comparison may therefore help distinguish biologically mediated color removal from direct oxidative decolorization.

Biological treatment provides an alternative approach in which microorganisms, microbial communities or extracellular enzymes interact with dye molecules through biosorption, bioaccumulation or enzymatic transformation. Bacteria may reduce azo bonds through azoreductase-mediated electron transfer, particularly when suitable electron donors and low-oxygen conditions are available. However, some bacterial cultures and microbial consortia can also transform azo dyes under aerobic or sequential Oxygen-restricted–aerobic conditions [7–10]. The initial reductive stage may remove the chromophore by cleaving the azo bond, whereas subsequent aerobic metabolism can assist in the degradation of the aromatic products. This dependence on oxygen availability is important because decolorization observed under Oxygen-restricted conditions does not necessarily indicate complete mineralization.

Bacterial species belonging to the genera *Bacillus*, *Enterococcus*, *Pseudomonas* and related groups have been investigated for azo-dye treatment. *Bacillus subtilis* is of particular interest because of its environmental adaptability, metabolic versatility, and potential to produce oxidoreductive enzymes. An indigenous strain of *B. subtilis* isolated from dye-contaminated sludge has previously demonstrated the

ability to decolorise simulated wastewater containing Congo Red, although its performance was affected by microbial growth kinetics and inhibition associated with dye transformation products [11]. Similarly, *Enterococcus* strains have been used to remove Acid Orange 7 and Direct Black 38 via a combination of biosorption and enzymatic transformation [5,9]. These findings indicate that bacterial treatment efficiency cannot be assumed to be identical across all azo dyes, as differences in molecular dimensions, the number of azo bonds, sulfonation, aromatic substitution, and solution chemistry may affect cellular uptake, surface interactions, and enzyme accessibility.

Fungi represent another important group of organisms used in dye remediation. White-rot fungi produce extracellular ligninolytic enzymes, including laccase, lignin peroxidase and manganese peroxidase. These enzymes possess relatively broad substrate specificity and can transform aromatic compounds that may be resistant to conventional bacterial treatment. White-rot fungal systems have achieved substantial decolorization of Congo Red and other azo dyes through a combination of adsorption onto fungal biomass and enzymatic transformation [12–14]. Nevertheless, the relative contribution of biosorption and chemical degradation varies among fungal species and treatment conditions. Incomplete transformation may also produce intermediates that retain biological toxicity, emphasising the need to avoid interpreting the disappearance of color as definitive evidence of detoxification.

Yeasts and naturally occurring microbial communities may also contribute to dye removal. Yeast cells can remove dyes through mechanisms including biosorption, bioaccumulation and, in some systems, biodegradation [15]. In contrast to the use of a defined bacterial or fungal culture, pond water and soil contain diverse microbial communities whose collective metabolic activity may support broader degradation pathways. Natural microbial treatments may therefore provide useful information regarding the potential environmental attenuation of dye contamination. However, their composition is generally variable, and treatment performance may be influenced by the source of the sample, nutrient availability, pH, oxygen conditions and competition between microorganisms. Comparing aerobic and Oxygen-restricted soil systems is particularly relevant because azo-bond reduction is often favoured under oxygen-limited conditions, whereas the degradation of the resulting aromatic compounds may require oxygen-dependent reactions.

The three dyes selected in the present study-Congo Red, Direct Black 38 and Acid Orange 7-provide a suitable basis for comparative investigation because they differ in molecular size, number of azo linkages and aromatic structure. Congo Red is a direct diazo dye whose decolorization by bacteria and white-rot fungi has been widely investigated [11–14]. Direct Black 38 is a structurally complex benzidine-based dye for which microbial azo-bond cleavage and the formation of aromatic intermediates have been documented [3–5]. Acid Orange 7, also known as Orange II, is a monoazo dye frequently used as a model pollutant in biological and oxidative treatment studies [6–10]. Direct comparison of these three dyes under the same simplified treatment and monitoring conditions remains useful because it can reveal whether apparent treatment effectiveness depends on dye structure as well as on the selected remediation system.

Accordingly, the present study evaluates the comparative decolorization behaviour of Congo Red, Direct Black 38 and Acid Orange 7 under a range of biological, natural and chemical treatment conditions. The systems investigated include white-rot fungi, *Bacillus subtilis*, yeast, pond water, aerobic and Oxygen-restricted soil-water mixtures, hydrogen peroxide, and a reverse-osmosis water control. Changes in color intensity and pH are monitored over seven days, with additional measurements during the first 24 hours to capture rapid initial responses. The study aims to determine which treatment conditions produce the greatest apparent color removal, examine differences in the responses of the three dyes and assess the influence of treatment type and oxygen availability.

METHODOLOGY

Experimental Design

A comparative experiment was conducted to investigate the apparent decolorization of Congo Red, Direct Black 38 and Acid Orange 7 under a range of biological, natural and chemical treatment conditions. For each dye, the treatment systems included reverse-osmosis (RO) water as a control, pond water, hydrogen peroxide, aerobic soil, Oxygen-restricted soil, aerobic yeast, Oxygen-restricted yeast, white-rot fungi and Bacillus subtilis.The experimental design compared the responses of Congo Red, Direct Black 38, and Acid Orange 7 to the biological and chemical treatments. Treatment volumes and stock concentrations were recorded for the respective experimental conditions. Oxygen-restricted conditions were established by covering the treatment vessels with airtight lids. These conditions were intended to restrict oxygen exchange; however, dissolved oxygen was not directly measured. Changes in pH and color intensity were monitored over seven days with an experimental temperature of 22.2℃ ± 1°, with additional measurements during the first 24 h to capture rapid initial responses. Also, a main part of this experiment was maintaining the Oxygen-restricted conditions which was done by sealing the container with an air tight lid to make it nominally oxygen-restricted.

Preparation of Treatment Solutions

Treatment solutions were prepared in individually labelled glass containers. The control contained 200 mL of RO water, while the pond-water treatment contained 200 mL of pond water collected from a local source. The hydrogen peroxide treatment contained 190 mL of water and 10 mL of 0.88M hydrogen peroxide solution. Soil treatments were prepared by combining 20 g of soil with 180 mL of water. Separate aerobic and Oxygen-restricted soil treatments were prepared. Yeast treatments contained 200 mL of water, one-half packet of Red Star Yeast, which was around 7g, and one teaspoon of sugar, with separate aerobic and Oxygen-restricted conditions. The fungal treatment contained 200 mL of water inoculated with 1 mL of white-rot fungal culture. The bacterial treatment contained 200 mL of water inoculated with one commercial capsule containing Bacillus subtilis from Supersmart with a dosage of 3 billion CFU for one. The capsule was opened, and its contents were directly dispersed into the experimental mixture without prior cultivation or pre-culture. All treatments including fungal, bacterial, yeast, soil, and pond water were incubated at a controlled temperature of 22.2°C ± 1.0°C, as mentioned in section 2.1, throughout the seven-day experimental period.

Preparation of Azo Dye Stock Solutions

Three azo-dye stock solutions were prepared before the experiment. Direct Black 38 was prepared by dissolving 1.00 g of dye in 100 mL of water. Congo Red was prepared by dissolving 500 mg in 50 mL of water, and Acid Orange 7 was prepared by dissolving 100 mg in 10 mL of water. Each stock, therefore, had a nominal concentration of 10 mg mL^{-1}. Within each dye series, the same volume of stock solution was added to each treatment: 0.75 mL Direct Black 38, 0.5 mL for Congo Red, and 0.25 mL for Acid Orange 7. This standardised the starting amount within each dye series but did not make the starting dye amount identical across the three different dyes.

Experimental Procedure and Monitoring

Immediately after adding the dye, baseline measurements were collected for each treatment. Initial pH was measured using pH indicator strips, and an overhead photograph was taken to record the initial appearance and color intensity of each sample. Samples were monitored for seven days. During the first 24 h, measurements were collected at 2, 4, 8 and 24 h. Thereafter, measurements were collected once per day for the remainder of the study. At each sampling point, pH was recorded, and an overhead photograph was captured. Photographs were taken from directly above the containers under consistent lighting and positioning conditions to reduce variability between measurements.

Image-Based Analysis of Color Change

Photographs were analysed using a custom web application developed in Replit. The software compared image-derived color-intensity values at each time point with the corresponding baseline image, generating estimates of percentage color removal over time.. The color change was assessed using images of the samples before and after treatment. The resulting image estimates were used to compare apparent changes in color among treatments. Since the analysis was based on images rather than UV–Vis spectrophotometry, the measurements should be estimates of color change rather than direct measurements of dye change. The application converted the measured light-intensity values into a relative optical-density measure using the average rate of change. The percentage color change was calculated by comparing the image-derived color-intensity value at each time point with the initial value recorded immediately after dye addition (T0). The initial image-derived color-intensity value was defined as the baseline, and the value obtained at a later time point was defined as the comparison value. Percentage color change was calculated using the following equation:

$$\%Color\ Change = \frac{I0 - It}{I0} * 100\%$$

where ($I0$) is the initial image-derived color-intensity value at baseline, which was the starting time(, and (It) is the image-derived color-intensity value at the later time point being compared.

Data Analysis

For each dye and treatment condition, pH and image-derived color-removal values were recorded at each sampling time. Data were organised by dye type, treatment condition, and experimental time. Trends were compared to identify treatments associated with the greatest apparent decolorization and to examine

whether the responses differed among Congo Red, Direct Black 38 and Acid Orange 7. Each treatment condition was evaluated in a single experimental trial (n = 1). Accordingly, the results are descriptive and do not have statistical testing or estimates of replicate variability. The observed differences should therefore be interpreted as base comparisons requiring confirmation through replicated experiments. The pH was evaluated as a supporting indicator of changes in the chemical environment of each treatment and was not used as a direct measure of dye degradation or decolorization.Throughout the analysis, visible color loss is described as decolorization or apparent color removal. Because degradation products were not directly identified and complete mineralization was not measured, color loss was not treated as definitive evidence of complete biodegradation or detoxification.

RESULTS

The effectiveness of the different treatment conditions was evaluated by monitoring both pH and percentage color change over the experimental period. Measurements were collected for Congo Red, Acid Orange 7 and Direct Black 38 immediately following dye addition (T0), at 2, 4, 8 and 24 h, and daily thereafter through Day 6, representing a seven-day experimental period from T0 to Day 6. Treatments included a reverse-osmosis water control, pond water, hydrogen peroxide, soil, yeast, fungi and bacteria, with selected biological treatments additionally maintained under oxygen-restricted conditions. Considerable differences were observed between the treatment conditions and among the three azo dyes. Overall, Direct Black 38 exhibited the greatest color change, Congo Red demonstrated moderate color change under a limited number of biological conditions, and Acid Orange 7 exhibited comparatively little color change throughout the experiment.

Congo Red

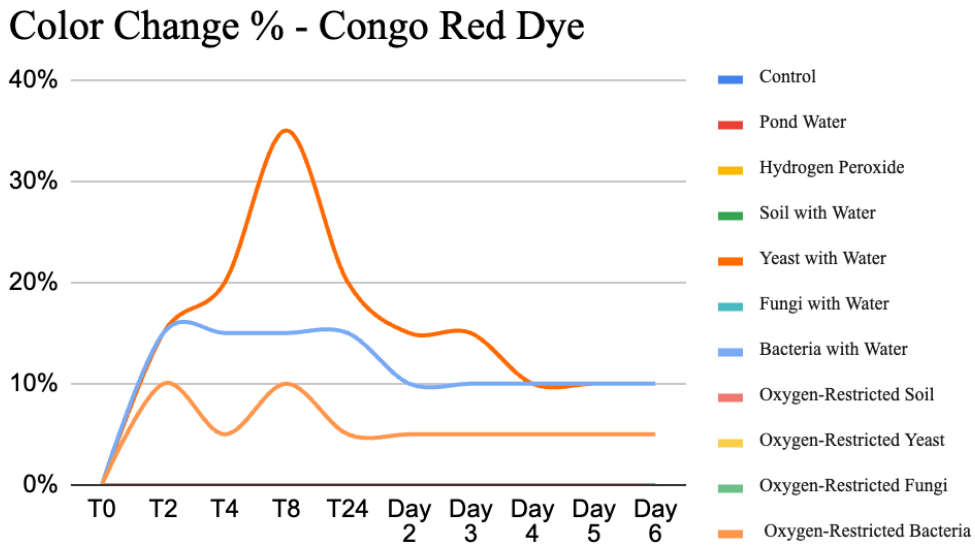


Figure 1. *Image-derived percentage colour change of Congo Red from baseline (T0) through Day 6 under the different treatment conditions. Treatments comprised the RO-water control, pond water, hydrogen peroxide, aerobic soil, aerobic yeast, white-rot fungi, aerobic Bacillus subtilis, oxygen-restricted soil, oxygen-restricted yeast and oxygen-restricted B. subtilis. T0 represents the initial measurement immediately after dye addition. Measurements were collected at 2, 4, 8, and 24 hours and daily thereafter through Day 6.*

Congo Red exhibited measurable color change primarily in the yeast and bacterial treatments (**Figure 1**). The aerobic yeast treatment produced the greatest response, with color change increasing from 0% at T0 to 15% after 2 h, 20% after 4 h and a maximum of 35% after 8 h. Following this maximum, the measured color change decreased to 20% at 24 h and ultimately stabilised at 10% from Day 4 through Day 6.

Aerobic bacteria also produced measurable changes in Congo Red. color change reached 15% after 2 h and remained at 15% through 24 h before declining to 10% for the remainder of the experiment. Oxygen-restricted bacteria demonstrated a smaller response, reaching a maximum observed color change of 10%. In contrast, no measurable color change was detected in the control, pond water, hydrogen peroxide, aerobic or Oxygen-restricted soil, fungal or Oxygen-restricted yeast treatments.

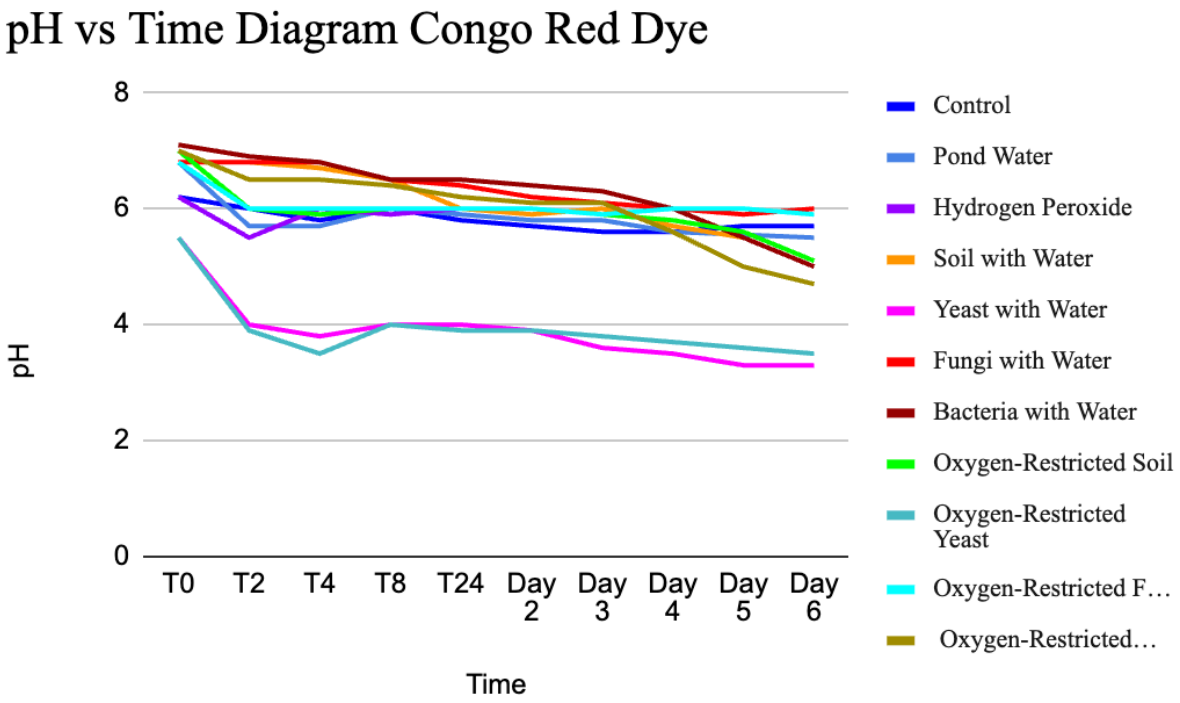


Figure 2. *Changes in pH of Congo Red from the initial measurement (T0) through Day 6 under the different treatment conditions. Treatments included the RO-water control, pond water, hydrogen peroxide, soil, yeast, fungi and bacteria under aerobic and oxygen-restricted conditions. T0 represents the initial measurement taken immediately after dye addition.*

Distinct changes in pH accompanied several of the biological treatment responses over the experimental period (**Figure 2**). The aerobic yeast treatment underwent particularly rapid acidification, decreasing from an initial pH of 5.5 to 4.0 within 2 h and reaching 3.3 by Day 6. Aerobic bacteria decreased more gradually from pH 7.1 to 5.0, while Oxygen-restricted bacteria decreased from 7.0 to 4.7. Comparatively little change occurred in the control and hydrogen peroxide treatments, which ended at pH 5.7 and 5.9, respectively.

Acid Orange 7

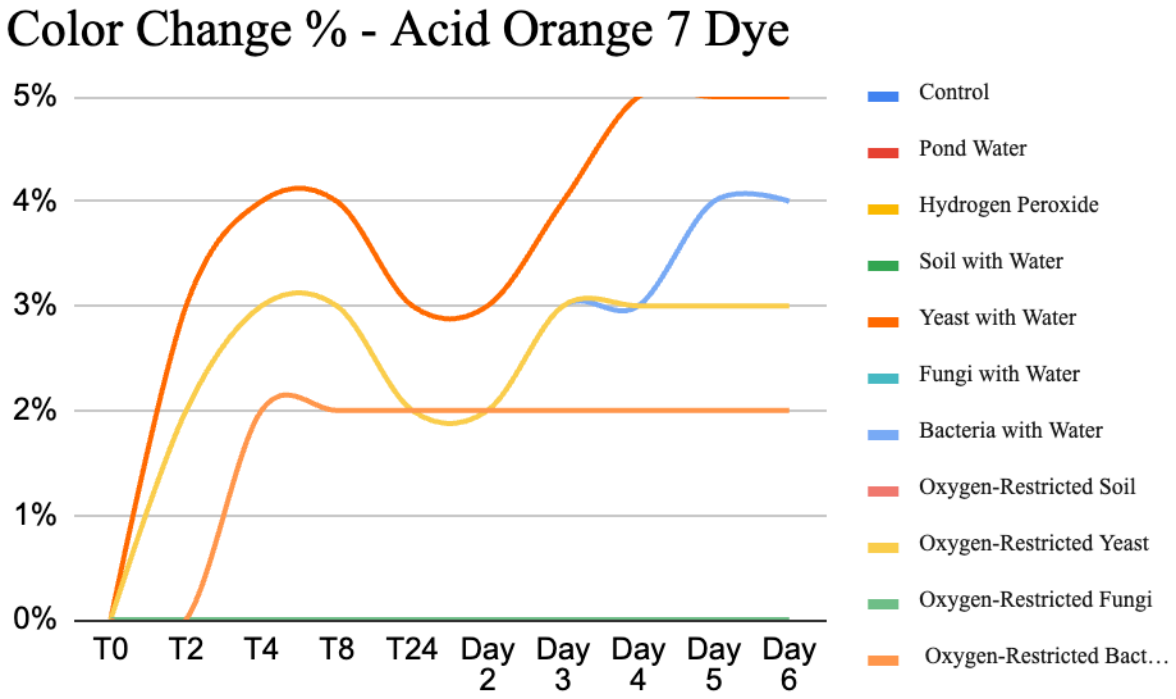


Figure 3. *Image-derived percentage colour change of Acid Orange 7 from baseline (T0) through Day 6 under the different treatment conditions. Treatments comprised the RO-water control, pond water, hydrogen peroxide, aerobic soil, aerobic yeast, white-rot fungi, aerobic Bacillus subtilis, oxygen-restricted soil, oxygen-restricted yeast and oxygen-restricted B. subtilis. T0 represents the initial measurement immediately after dye addition.*

Acid Orange 7 was the least responsive of the three dyes based on percentage color change (**Figure 3**). Aerobic yeast produced the greatest measured change, but values remained low throughout the experiment. color change increased from 0% at T0 to 3% after 2 h and reached a maximum of 5% from Day 4 through Day 6. Aerobic bacteria reached a maximum color change of 4% on Days 5 and 6. Oxygen-restricted yeast reached a maximum observed value of 3%, while Oxygen-restricted bacteria reached approximately 2%. No measurable color change occurred in the control, pond water, hydrogen peroxide, soil or fungal treatments.

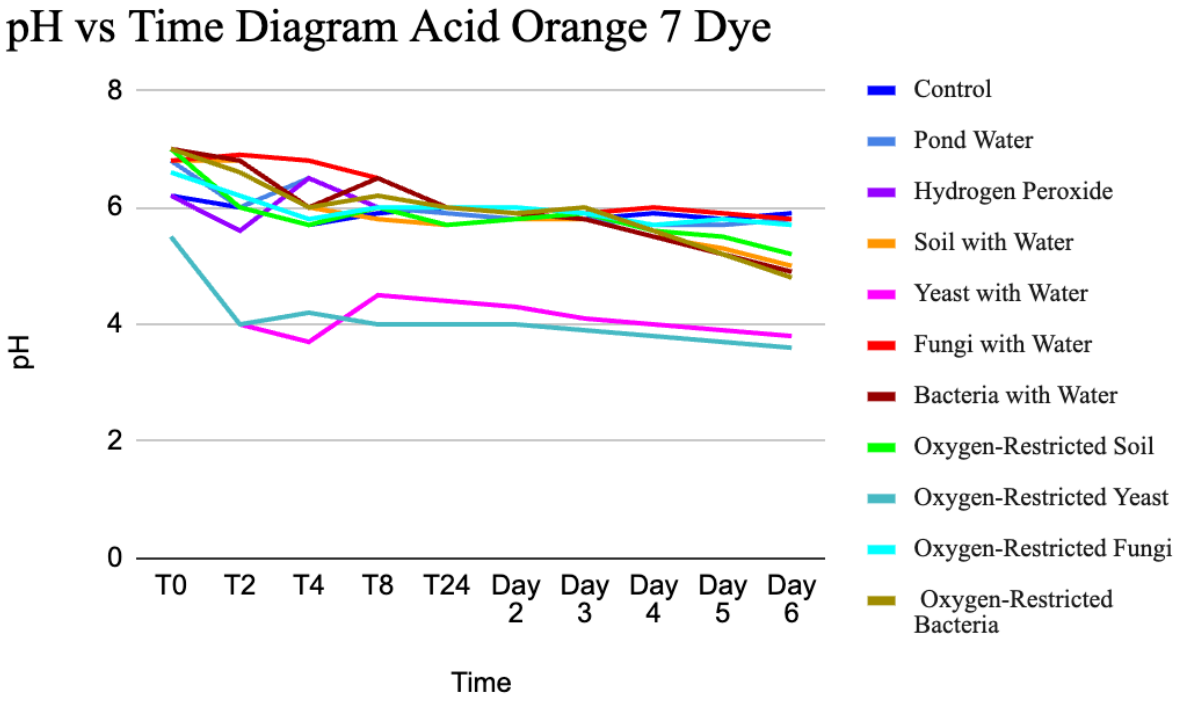


Figure 4. *Changes in pH of Acid Orange 7 from the initial measurement (T0) through Day 6 under the different treatment conditions. Treatments included the RO-water control, pond water, hydrogen peroxide, soil, yeast, fungi and bacteria under aerobic and oxygen-restricted conditions. T0 represents the initial measurement taken immediately after dye addition.*

Despite the limited color change, several Acid Orange 7 biological treatments underwent substantial changes in pH (**Figure 4**). Aerobic yeast decreased from an initial pH of 5.5 to 4.0 after 2 h and ultimately reached 3.8 on Day 6. Aerobic and Oxygen-restricted bacterial treatments decreased from an initial pH of 7.0 to final values of 4.9 and 4.8, respectively. In contrast, the control remained comparatively stable, changing from pH 6.2 to 5.9, while hydrogen peroxide decreased only from 6.2 to 5.8. Thus, substantial changes in solution pH did not necessarily correspond with large changes in Acid Orange 7 color intensity.

Direct Black 38

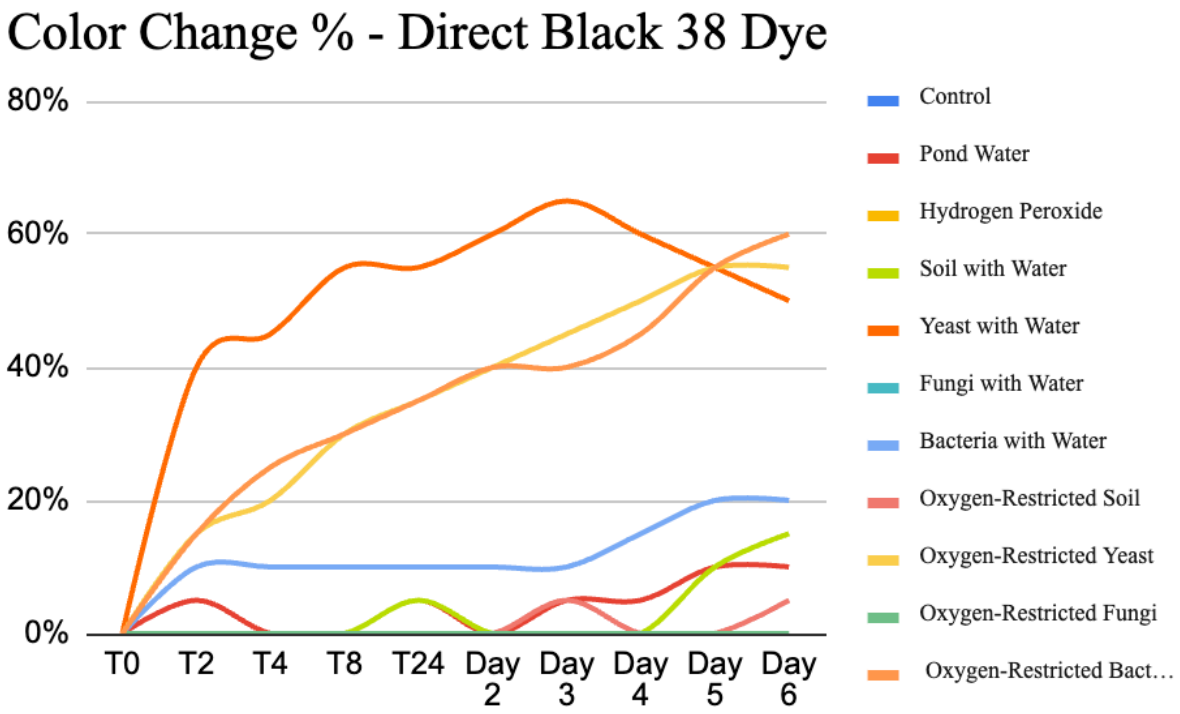


Figure 5. *Image-derived percentage colour change of Direct Black 38 from baseline (T0) through Day 6 under the different treatment conditions. Treatments comprised the RO-water control, pond water, hydrogen peroxide, aerobic soil, aerobic yeast, white-rot fungi, aerobic Bacillus subtilis, oxygen-restricted soil, oxygen-restricted yeast and oxygen-restricted B. subtilis. T0 represents the initial measurement immediately after dye addition.*

Direct Black 38 demonstrated the greatest overall color response and the clearest differences among biological treatment conditions (**Figure 5**). Aerobic yeast produced the strongest and most rapid color change. color change increased from 0% at T0 to 40% after only 2 h, 45% after 4 h and 55% after 8 h. It reached 60% on Day 2 and a maximum observed value of 65% on Day 3. Following this maximum, the measured color change decreased slightly, reaching 50% by Day 6.

Oxygen-restricted yeast also produced substantial color change, although its response developed more gradually. color change increased from 15% after 2 h to 20% after 4 h, 30% after 8 h and 35% after 24 h. It continued increasing over subsequent days, ultimately reaching 55% on Days 5 and 6. Oxygen-restricted bacteria demonstrated another strong response and exhibited a progressive increase in color change throughout the experiment. Values increased from 15% after 2 h to 25% after 4 h, 30% after 8 h and 35% after 24 h. color change subsequently increased to 40% on Days 2 and 3, 45% on Day 4, 55% on Day 5 and a maximum of 60% on Day 6. Aerobic bacteria produced a smaller response, reaching a maximum of 20% on Days 5 and 6.

The remaining Direct Black 38 treatments exhibited considerably smaller changes. Aerobic soil reached 15% by Day 6, while pond water reached 10%. Oxygen-restricted soil exhibited only minor changes, reaching 5% at selected time points. No measurable color change occurred in the control, hydrogen peroxide or fungal treatment.

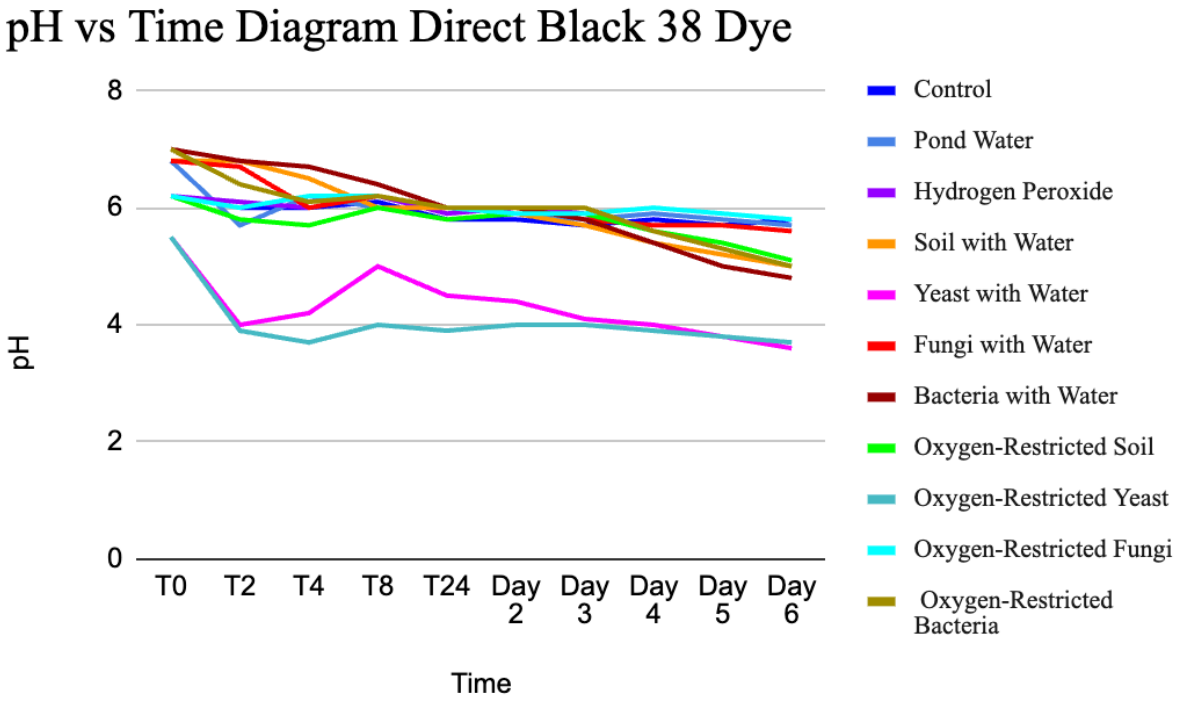


Figure 6. *Changes in pH of Direct Black 38 from the initial measurement (T0) through Day 6 under the different treatment conditions. Treatments included the RO-water control, pond water, hydrogen peroxide, soil, yeast, fungi and bacteria under aerobic and oxygen-restricted conditions. T0 represents the initial measurement taken immediately after dye addition.*

Direct Black 38 also demonstrated substantial treatment-dependent changes in pH (Figure 6). Aerobic yeast decreased from pH 5.5 at T0 to 4.0 after 2 h and ultimately reached 3.6 on Day 6. Oxygen-restricted yeast decreased from 5.5 to 3.7. Aerobic bacteria decreased from an initial pH of 7.0 to 4.8, while Oxygen-restricted bacteria decreased from 7.0 to 5.0. The control and hydrogen peroxide treatments remained comparatively stable, both ending at pH 5.8.

Comparison of Treatment Effectiveness Across Azo Dyes

Comparison across all three dyes showed that the extent of color change varied considerably with both treatment condition and dye type. Direct Black 38 was substantially more responsive than Congo Red or Acid Orange 7. Under aerobic yeast treatment, the maximum observed color changes were 65% for Direct Black 38, 35% for Congo Red and 5% for Acid Orange 7.

The effect of aerobic and oxygen-restricted conditions was most apparent for Direct Black 38. Oxygen-restricted bacteria reached 60% color change by Day 6, compared with 20% for aerobic bacteria at the same time point. Similarly, Oxygen-restricted yeast reached 55% by Day 6, slightly exceeding the 50% measured for aerobic yeast at the end of the experiment. However, aerobic yeast produced a considerably faster initial response, reaching 40% color change within the first 2 h and a maximum observed value of 65% by Day 3.

The control exhibited no measurable color change for any of the three dyes. Hydrogen peroxide and the fungal treatment similarly produced no measurable color change throughout the experiment. Pond water and soil demonstrated only limited changes, primarily with Direct Black 38. Under the conditions and duration of the present experiment, the largest color changes were therefore observed in the yeast and bacterial treatments.

Changes in pH were also treatment dependent but did not consistently correspond with the magnitude of color change. Yeast treatments generally underwent rapid decreases in pH, while bacterial treatments showed more gradual decreases. Acid Orange 7 demonstrated substantial decreases in pH in several biological treatments despite exhibiting only minimal color change. Therefore, changes in pH and measured color intensity showed different patterns across the treatment systems.

Overall, Direct Black 38 exhibited the greatest apparent response to the biological treatments tested, particularly yeast and bacteria. Congo Red showed a smaller and more variable response, whereas Acid Orange 7 showed comparatively little change in color intensity during the experimental period.

DISCUSSION

The present study compared the response of three azo dyes namely Congo Red, Direct Black 38 and Acid Orange 7 to a range of biological, natural and chemical treatment conditions. The results showed that treatment effectiveness varied considerably according to both the type of dye and the treatment used. Among the three dyes, Direct Black 38 showed the greatest apparent decolorization, particularly in yeast and bacterial treatments. Congo Red exhibited a more moderate response, while Acid Orange 7 remained comparatively resistant under most experimental conditions. These differences suggest that a treatment capable of removing the color of one azo dye may not necessarily be equally effective for another. Also, The mechanistic explanations discussed below are interpretations based on previously reported microbial pathways and were not directly measured in the present experiment.

Effect of Biological Treatments on Direct Black 38

Direct Black 38 showed the strongest response to biological treatment. The greatest color changes were observed in treatments containing yeast and bacteria, whereas the control and several non-biological treatments showed comparatively little change. In particular, both aerobic and oxygen-restricted yeast

treatments produced substantial changes in color intensity. The oxygen-restricted bacterial treatment also showed a strong and progressive response over time.

The rapid response observed in the yeast treatments suggests that interactions between the yeast cells and the dye contributed to the reduction in visible color. Several mechanisms could potentially contribute to this behaviour, including biosorption of dye molecules onto the cell surface, bioaccumulation and microbial transformation. Therefore, the observed color removal should not automatically be interpreted as complete biodegradation of Direct Black 38.

The response of Direct Black 38 under oxygen-restricted bacterial conditions is also noteworthy. Azo dyes contain one or more azo bonds (–N=N–), and previous studies have shown that microbial reduction of these bonds can be favoured under oxygen-limited conditions [3–5]. Reduction of the azo bond disrupts the chromophore responsible for visible color and can therefore result in substantial decolorization. The stronger response observed for oxygen-restricted bacteria compared with aerobic bacteria may be consistent with this mechanism. However, the present experiment did not directly identify molecular degradation products. Consequently, azo-bond cleavage cannot be confirmed from color measurements alone.

Response of Congo Red

Congo Red exhibited a less pronounced response than Direct Black 38. Among the biological treatments, aerobic yeast produced the greatest initial apparent color change, with a relatively rapid increase during the first several hours. However, the measured color-removal value subsequently decreased rather than continuing to increase throughout the experiment.

This non-linear trend is important when interpreting the findings. If decolorization resulted only from irreversible chemical destruction of the dye molecules, a more continuous increase in color removal might be expected. Instead, changes in turbidity, microbial growth, settling of suspended material, aggregation of dye molecules or small variations during image collection may have influenced the calculated color intensity. Dye molecules may also have temporarily interacted with microbial biomass before being redistributed within the solution.

Previous studies have demonstrated that both bacterial and fungal systems can decolorise Congo Red under suitable experimental conditions [11–14]. The more limited response observed in the present experiment therefore does not necessarily conflict with the literature. Differences in microbial concentration, nutrient availability, incubation conditions and analytical methods may contribute to differences in apparent treatment efficiency.

Therefore, although the Congo Red results indicate that the yeast treatment affected the appearance of the dye solution, the experiment does not provide sufficient evidence to conclude that the initial reduction in color represented permanent degradation of the dye. This observation illustrates the importance of examining the complete time-dependent response rather than considering only the maximum color-removal value.

Response of Acid Orange 7

Acid Orange 7 showed the lowest apparent decolorization of the three dyes investigated. Only small changes in color intensity were observed even in the yeast and bacterial treatments. This occurred despite measurable changes in pH within several biologically active treatment systems.

This result provides an important comparison with Direct Black 38. Although the same general types of biological treatments were applied to all three dyes, their responses were substantially different. The findings therefore suggest that biological remediation efficiency is strongly dependent on the characteristics of the individual dye. Differences in molecular structure, molecular size, number of azo bonds, functional groups and interactions with microbial cells may influence how readily a dye responds to treatment.

Previous studies have reported substantial biodegradation or decolorization of Acid Orange 7 using bacterial systems under more controlled or optimised conditions [6–10]. The considerably smaller response observed in the present experiment may therefore reflect differences in microbial species, treatment duration, nutrient availability, dye concentration, oxygen conditions or analytical technique.

The limited response of Acid Orange 7 also demonstrates why the behaviour of one azo dye should not be generalised to the entire class. Although all three compounds contain azo groups, they are chemically distinct molecules and may interact differently with microorganisms and their enzymes.

Comparison Between the Three Dyes

The most important overall finding was the clear difference in response among the three azo dyes. Direct Black 38 produced the largest treatment-dependent color changes, Congo Red showed an intermediate and more variable response, and Acid Orange 7 was comparatively resistant.

These differences support the idea that successful azo-dye remediation depends not only on selecting a microorganism but also on the chemical characteristics of the target dye [19,20]. The term “azo dye” describes a broad chemical class rather than a group of compounds with identical environmental behaviour [15,16,18]. Even dyes containing the same azo functional group can differ considerably in molecular structure and susceptibility to biological treatment [21,22].

The experimental results also differed from several published studies in which bacterial and fungal treatments produced much greater levels of decolorization or biodegradation [5,7–14,17,27]. In the present study, yeast and bacteria produced the greatest visible responses, particularly for Direct Black 38, whereas the white-rot fungal treatment produced no measurable color change.

These differences should not be interpreted as contradicting previous research. Published studies often use selected microbial strains, controlled nutrient media, optimised temperatures and pH values, higher microbial concentrations, longer treatment periods and spectrophotometric measurements. In contrast, the present experiment was designed as a simplified comparative study. Furthermore, the current study

measured image-derived color change rather than directly measuring molecular degradation. Direct numerical comparison between the present color-removal values and published biodegradation efficiencies should therefore be made cautiously.

Overall, the findings suggest that textile wastewater treatment strategies may need to be selected according to the dyes present rather than assuming that a single biological treatment will perform equally well for all azo contaminants [23,24].

Treatment Mechanisms and Relationship with pH

One of the clearest observations from the experiment was that changes in pH did not always correspond with changes in color intensity. Yeast treatments showed particularly rapid decreases in pH. For example, the pH of the Congo Red aerobic yeast treatment decreased from approximately 5.5 to 4.0 within two hours and continued to decrease during the experimental period. Bacterial treatments generally showed slower but more sustained decreases in pH.

The reduction in pH suggests that microbial activity altered the chemical environment of the solutions. In yeast-containing treatments, metabolism of the supplied sugar may have contributed to the production of acidic metabolites and the observed acidification. Bacterial metabolism may similarly have generated compounds capable of changing solution pH.

However, pH change alone did not indicate effective dye removal. This was particularly evident for Acid Orange 7, where substantial acidification occurred in several biological treatments despite very little corresponding color change. Therefore, pH should be considered an indicator of changes occurring within the treatment system rather than a direct measurement of azo-dye degradation.

This distinction is important because microorganisms can remain metabolically active without necessarily degrading the dye. Conversely, visible color may decrease through processes such as adsorption or precipitation without substantial microbial degradation. Considering both pH and color measurements therefore provided a more informative assessment than either measurement alone.

Other Treatment Systems

The limited apparent response of the white-rot fungal treatment was unexpected because white-rot fungi have frequently been reported to remove synthetic dyes through extracellular ligninolytic enzymes, including laccase and peroxidases [12–14]. However, the absence of substantial decolorization in the present experiment does not demonstrate that white-rot fungi are generally ineffective for azo-dye treatment.

Fungal activity depends strongly on environmental conditions, including temperature, pH, nutrient availability, fungal growth stage, inoculum concentration and treatment duration. The simplified experimental conditions used in this study may therefore not have been optimal for fungal establishment or enzyme production. The result should consequently be interpreted as showing that the fungal treatment

did not produce measurable decolorization under the specific conditions tested, rather than concluding that white-rot fungi cannot degrade these dyes.

Hydrogen peroxide similarly showed little visible improvement compared with the control. Hydrogen peroxide was included as a chemical oxidation treatment because oxidative reactions can contribute to destruction of dye chromophores. However, hydrogen peroxide alone may show limited effectiveness under some conditions, and its activity may be enhanced when combined with ultraviolet irradiation, catalysts or other advanced oxidation processes [6]. The limited response observed in this experiment therefore applies specifically to the treatment concentration and conditions used in the present study.

The soil treatments presented an additional challenge because the soil itself substantially changed the color and turbidity of the solutions. Both aerobic and oxygen-restricted soil mixtures appeared dark brown or black, making it difficult to distinguish changes caused by the azo dye from those produced by suspended soil particles.

Consequently, photographic color analysis of the soil treatments should be interpreted cautiously. Changes in image-derived color intensity may have resulted partly from sedimentation, suspended particles or changes in soil turbidity rather than actual removal of the dye. This represents an important experimental limitation because the same image-analysis method may not provide equally reliable measurements for relatively clear dye solutions and highly turbid soil suspensions.

Pond water also contained naturally occurring microbial communities but produced comparatively little visible color change under the conditions investigated. This may indicate that the concentration or composition of the indigenous microbial population was insufficient to produce substantial decolorization during the experimental period. However, the microbial composition of the pond water was not determined in the present study.

Limitations of the Study

Several limitations should be considered when interpreting these results. Colour change was estimated from digital photographs rather than measured using UV–Vis spectrophotometry, so the results represent apparent decolorization and may be affected by lighting, reflections, suspended particles, microbial biomass and solution turbidity. The use of pH indicator strips also limited measurement precision. In addition, oxygen-restricted conditions were created using sealed containers, but dissolved oxygen was not directly measured; therefore, these conditions cannot be confirmed as fully anaerobic. Each treatment was conducted once (n = 1), preventing assessment of experimental variability or statistical comparison. Finally, degradation products and toxicity were not assessed, so the observed colour loss does not establish complete biodegradation, mineralization or detoxification.

Implications and Future Work

Despite these limitations, the study demonstrates that relatively simple biological systems can produce clearly different responses when applied to different azo dyes. Yeast and bacterial treatments showed the greatest potential under the conditions investigated, particularly for Direct Black 38. In contrast, the limited response of Acid Orange 7 demonstrates that successful treatment cannot be assumed simply because a pollutant belongs to the azo-dye class [21,27].

Future studies should use UV-Visible spectrophotometry to quantify changes in dye concentration more directly and should include multiple experimental replicates. Treatment variables such as microbial concentration, temperature, pH, oxygen availability and nutrient concentration could also be more precisely controlled. The difference between aerobic and oxygen-restricted bacterial responses observed for Direct Black 38 suggests that oxygen availability would be particularly valuable to investigate as an independent experimental variable.

Longer treatment periods could also determine whether treatments showing limited initial responses, particularly white-rot fungi, eventually produce greater decolorization. Identification and toxicity assessment of degradation products would also be necessary to establish whether visible color removal corresponds to actual molecular degradation and environmental detoxification [26,27]. Alternative non-biological or bio-assisted approaches, such as low-cost natural adsorbents [25] or functionalized nanoparticles [29], may also be integrated to enhance systemic treatment efficiency.

Overall, the findings indicate that biological treatment has potential as an approach for removing azo dyes from contaminated water, but its effectiveness is highly dependent on both the microorganism and the dye being treated [23,28]. Direct Black 38 responded most strongly to biological treatment, Congo Red showed a moderate and more variable response, and Acid Orange 7 was comparatively resistant. These findings provide a useful basis for further investigation of low-cost biological approaches to textile wastewater remediation [28].

CONCLUSION

This study compared the apparent removal of Congo Red, Direct Black 38 and Acid Orange 7 under biological, natural and chemical treatment conditions. The findings indicate that treatment response depended strongly on both the dye and the treatment system. Direct Black 38 exhibited the greatest apparent decolorization, particularly in treatments involving yeast and Oxygen-restricted bacteria. Congo Red showed a more moderate and variable response, whereas Acid Orange 7 remained comparatively resistant under the conditions tested.The pH measurements also showed that substantial changes in solution pH could occur without a corresponding decrease in visible colour.

An important outcome of the study is the need to distinguish decolorization from complete biodegradation [26]. Although several treatments produced visible color loss, the experimental methods did not identify degradation products or determine whether the treated solutions became less toxic [26,27]. Accordingly, the results support further investigation of microbial treatment as a potentially low-cost and sustainable approach to azo-dye remediation [28], but they do not by themselves demonstrate complete or environmentally safe degradation.

Future work should include independent experimental replicates, calibrated UV-Visible spectrophotometry, improved control of microbial concentration and oxygen availability, and chemical or toxicity analysis of treatment products. These improvements would allow the mechanisms responsible for color removal to be assessed more directly and would help determine whether the most promising treatments can be developed into reliable wastewater-remediation strategies [23,28].

REFERENCES

[1] Selvam, K., Swaminathan, K. and Chae, K.-S. (2003). Decolorization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp. *Bioresource Technology*, **88**(2), 115–119. doi: 10.1016/S0960-8524(02)00280-8.

[2] Coughlin, M.F., Kinkle, B.K. and Bishop, P.L. (2002). Degradation of Acid Orange 7 in an aerobic biofilm. *Chemosphere*, **46**(1), 11–19. doi: 10.1016/S0045-6535(01)00096-0.

[3] Manning, B.W., Cerniglia, C.E. and Federle, T.W. (1985). Metabolism of the benzidine-based azo dye Direct Black 38 by human intestinal microbiota. *Applied and Environmental Microbiology*, **50**(1), 10–15. doi: 10.1128/AEM.50.1.10-15.1985.

[4] Kumar, K., Devi, S.S., Krishnamurthi, K., Gampawar, S., Mishra, N., Pandya, G.H. and Chakrabarti, T. (2006). Decolorization, biodegradation and detoxification of benzidine-based azo dye. *Bioresource Technology*, **97**(3), 407–413. doi: 10.1016/j.biortech.2005.03.031.

[5] Bafana, A., Krishnamurthi, K., Devi, S.S. and Chakrabarti, T. (2008). Biological decolorization of C.I. Direct Black 38 by *Enterococcus gallinarum*. *Journal of Hazardous Materials*, **157**(1), 187–193. doi: 10.1016/j.jhazmat.2007.12.085.

[6] Aleboyeh, A., Moussa, Y. and Aleboyeh, H. (2005). Kinetics of oxidative decolorization of Acid Orange 7 in water by ultraviolet radiation in the presence of hydrogen peroxide. *Separation and Purification Technology*, **43**(2), 143–148. doi: 10.1016/j.seppur.2004.10.014.

[7] Coughlin, M.F., Kinkle, B.K. and Bishop, P.L. (2003). High-performance degradation of azo dye Acid Orange 7 and sulfanilic acid in a laboratory-scale reactor after seeding with cultured bacterial strains. *Water Research*, **37**(11), 2757–2763. doi: 10.1016/S0043-1354(03)00069-1.

[8] Lim, C.K., Bay, H.H., Aris, A., Abdul Majid, Z. and Ibrahim, Z. (2013). Biosorption and biodegradation of Acid Orange 7 by *Enterococcus faecalis* strain ZL: optimization by response-surface methodological approach. *Environmental Science and Pollution Research*, **20**(7), 5056–5066. doi: 10.1007/s11356-013-1476-5.

[9] Fan, J., Li, W., Zhang, B., Shi, W. and Lens, P.N.L. (2022). Unravelling the biodegradation performance and mechanisms of Acid Orange 7 by aerobic granular sludge at different salinity levels. *Bioresource Technology*, **357**, 127347. doi: 10.1016/j.biortech.2022.127347.

[10] Thung, W.-E., Ong, S.-A., Ho, L.-N., Wong, Y.-S., Ridwan, F., Lehl, H.K., Oon, Y.-L. and Oon, Y.-S. (2018). Biodegradation of Acid Orange 7 in a combined anaerobic up-flow membrane-less microbial fuel cell: Mechanism of biodegradation and electron transfer. *Chemical Engineering Journal*, **336**, 397–405. doi: 10.1016/j.cej.2017.12.028.

[11] Chaturvedi, A., Rai, B.N., Singh, R.S. and Jaiswal, R.P. (2021). A computational approach to incorporate metabolite inhibition in the growth kinetics of indigenous bacterial strain *Bacillus subtilis* MN372379 in the treatment of wastewater containing Congo Red dye. *Applied Biochemistry and Biotechnology*, **193**(7), 2128–2144. doi: 10.1007/s12010-021-03538-4.

[12] Wang, N., Chu, Y., Wu, F., Zhao, Z. and Xu, X. (2017). Decolorization and degradation of Congo Red by a newly isolated white-rot fungus, *Ceriporia lacerata*, from decayed mulberry branches. *International Biodeterioration & Biodegradation*, **117**, 236–244. doi: 10.1016/j.ibiod.2016.12.015.

[13] Chakraborty, S., Basak, B., Dutta, S., Bhunia, B. and Dey, A. (2013). Decolorization and biodegradation of Congo Red dye by a novel white-rot fungus *Alternaria alternata* CMERI F6. *Bioresource Technology*, **147**, 662–666. doi: 10.1016/j.biortech.2013.08.117.

[14] Kong, F., Wang, A., Cheng, H. and Liang, B. (2014). Accelerated decolorization of azo dye Congo Red in a combined bioanode–biocathode bioelectrochemical system with modified electrode deployment. *Bioresource Technology*, **151**, 332–339. doi: 10.1016/j.biortech.2013.10.027.

[15] Fundamentals of natural fibres and textiles. (2021). Duxford: Woodhead Publishing.

[16] Li, X., Zhu, L., Ding, X., Wu, X. and Wang, L. (2024). Climate change and the textile industry: The carbon footprint of dyes. Journal of Engineered Fibers and Fabrics, 19. doi: 10.1177/24723444231212954.

[17] Das, S., Cherwoo, L. and Singh, R. (2023). Decoding dye degradation: Microbial remediation of textile industry effluents. Biotechnology Notes, [online] 4, pp.64–76. doi:10.1016/j.biotno.2023.10.001.

[18] Fineotex Chemical Limited. (2024). 4 environmental issues caused by textile chemicals & their possible solutions. Fineotex Chemical Limited.

[19] Campbell, J. (2022). Azo compound (azo dye). EBSCO Research Starters: Chemistry.

[20] ScienceDirect Topics. Azo dye. Pharmacology, Toxicology and Pharmaceutical Science. Elsevier.

[21] Carrascal-Hernández, D. C., Orozco-Beltrán, E. J., Insuasty, D., Márquez, E. and Grande-Tovar, C. D. (2025). Systematic evaluation of biodegradation of azo dyes by microorganisms: Efficient species, physicochemical factors, and enzymatic systems. International Journal of Molecular Sciences, 26(16), 7973. doi: 10.3390/ijms26167973.

[22] Chen, H. (2006). Recent advances in azo dye degrading enzyme research. Current Protein & Peptide Science, 7(2), 101–111. doi: 10.2174/138920306776359786.

[23] Kumar, M., Mishra, A., Patel, S. K., et al. (2025). Environmental impacts and strategies for bioremediation of dye-containing wastewater. Bioengineering, 12(10), 1043. doi: 10.3390/bioengineering12101043.

[24] Schroecker, J. (2019). Why we need to get rid of azo dyes. Mogalixe.

[25] Aranda-Figueroa, M. G., Rodríguez-Torres, A., Rodríguez, A., Bolio-López, G. I., Salinas-Sánchez, D. O., Arias-Atayde, D. M., Romero, R. J. and Valladares-Cisneros, M. G. (2024). Removal of azo dyes from water using natural Luffa cylindrica as a non-conventional adsorbent. Molecules, 29(9), 1954. doi: 10.3390/molecules29091954.

[26] Rawat, D., Mishra, V. and Sharma, R. S. (2016). Detoxification of azo dyes in the context of environmental processes. Chemosphere, 155, 591–605. doi: 10.1016/j.chemosphere.2016.04.068.

[27] Goswami, D., Mukherjee, J., Mondal, C. and Bhunia, B. (2024). Bioremediation of azo dye: A review on strategies, toxicity assessment, mechanisms, bottlenecks and prospects. Science of The Total Environment, [online] 954, p.176426. doi:10.1016/j.scitotenv.2024.176426.

[28] Delta Remediation. (2025). Bioremediation for sustainable development goals. Delta Remediation.

[29] Vinayak, A. and Singh, G.B. (2022). Biodecolorization of reactive black 5 using magnetite nanoparticles coated Bacillus sp. RA5. SCPINM-2021, [online] 48, pp.1523–1526. doi:10.1016/j.matpr.2021.09.425.

[30] Ghori, F.A., Wu, Y., Lin, X., Yu, J., Chen, H., Jhatial, A.K., Zhou, Q., Sadannavar, M.K. and Xue, G. (2025). Impact of sodium dithionite salinity stress on hydrolysis of urea and total nitrogen removal in textile printing wastewater: Insights from anoxic-oxic-anoxic process. Process Safety and Environmental Protection, 200, 107333. doi: 10.1016/j.psep.2025.107333.