

Decolourization of direct red dye using isolates of *Bacillus* species

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ABSTRACT

Biodegradation offers an effective method in order to eliminate textile dyes from the environment. Direct Red, a widely used textile dye, poses significant environmental risks due to its complex structure and resistance to conventional remediation methods. Synthetic dyes, in particular, are more difficult to decolorize than natural dyes, making their treatment in industrial wastewater a global concern. Traditional physical and chemical approaches are often inefficient, but certain microorganisms can utilize dyes as a carbon or nitrogen source, providing a cost-effective bioremediation alternative. This study focused on isolating direct red dye-decolorizing bacteria, optimizing decolourization conditions, and analysing the outcomes. *Bacillus* species demonstrated significant potential, achieving up to 80% degradation of Direct Red dye within 24-48 hours at neutral pH and 28°C temperature. Factors such as temperature, pH, static and shaking conditions and media composition were examined to determine how they affected decolourization. UV spectrophotometric analysis revealed that the dye was decolourized, highlighting the potential of *Bacillus* species for bioremediation of textile dye pollution. Ultimately, this strategy has a lot of potential for advancing sustainable wastewater treatment technologies in future.

Keywords: Direct Red dye, *Bacillus* species, Decolourization..

1. INTRODUCTION

About 1,00,000 distinct commercial dyes are accessible on an industrial scale, and more than 7×10^5 metric tons of dye stuffs are manufactured worldwide each year. According to estimates, 10 % to 50% of these reactive dyes used when processing textiles are released as a wastewater discharge (Khan *et al.*, 2013). This changes the pH and other variables, giving the water bodies a vibrant colour. The usage of these water resources is restricted, and the ecology suffers as a result. The azo, anthraquinone, and triphenylmethane dyes are examples of synthetic dyes. Azo dyes represent a major group of dyes utilized at the industrial level (Jiang *et al.*, 2023). The induced or self-reductive Breakage of the azo bond is responsible for The production of toxic amines, known to be a major environmental problem due to their colour, bio-resistance, and possible toxicity to animals and humans (Robson *et al.*, 2012). Azo dyes are the most extensively used dye group in the textile industry. (Lacasse and Baumann *et al.*, 2004). It has been found that they are responsible for 60-70% of all dyestuff applied in the textile industry manufacture. Aromatic-based amines (AAs) can be released during the dermal, systemic, and bacterial biotransformation of azo dyes. AAs applied to the skin may may be taken up dermally to a large extent. (Korinith *et al.*, 2013). As intermediates in the manufacture of azo dyes, AAs are employed. Exposure to AAs through consumer products poses dangers to human health, notably because of the mutagenic and/or carcinogenic qualities of certain AAs. The harmful effects of aromatic amines (AAs) are because of the metabolic activation of the amino group, which can produce the reactive intermediate hydroxylamine, which has been observed to harm DNA and proteins. Out of the 896 azo dyes with defined chemical structures in our from the textile dye database, 426 (48%) are possible parent compounds at least one of the 22 controlled AAs, whereas the remaining 470 (52%) are solely converted to non-regulated AAs.

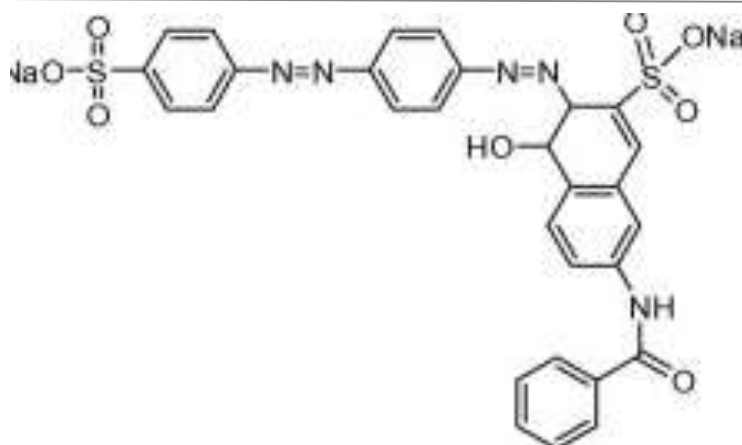


Fig. 1. Structure of the compound Direct Red dye (Harichandran *et al.*, 2016)

Dyes produced through synthetic methods are highly persistent in natural environments due to their chemical and photolytic stability, posing Eco toxicity risks and affecting the food chain. While water-soluble dyes have lower bioaccumulation potential, non-ionic dyes and pigments can accumulate significantly. Biological methods for dye decolourization, using microorganisms, are more economical, eco-friendly, and efficient compared to physicochemical approaches. Microbes decolorize dyes by adsorption or degradation, with microbial consortia demonstrating greater effectiveness than pure strains. Synthetic Azo dye degradation involves anaerobic and aerobic processes. Enzymes like azoreductase and laccase aid in dye breakdown, with both living and dead microbial cells being effective. The biosorption process, though potentially useful for dye recovery, faces practical challenges such as sludge disposal. Biodegradation, on the other hand, degrades organic dyes into fragments that must be fully mineralized into CO₂ and H₂O, offering a more natural solution for eliminating organic pollutants.

Fungi have proven effective in dye decolourization due to their enzymatic capabilities, including adsorption and degradation via enzymes like laccases and peroxidases. Despite achieving over 90% colour reduction under optimal conditions, fungi exhibit low pH stability, limiting their application. For example, the white-rot fungus *Ceriporiolacerata* degraded 90% of Congo red dye within 48 h. There has been limited research on yeast for dye decolourization and degradation, with most research focusing on biosorption. It is effective in decolorizing azo dyes because of its ability to accumulate dyes and heavy metals like lead and Cadmium (II) (Fairhead and Meyer.,2012), faster growth compared to filamentous fungi, and resilience under harsh conditions (Martorell *et al.*, 2012). Although wastewater sludge contains diverse yeast strains, they represent a small fraction of activated sludge microorganisms. Yeast utilizes adsorption and enzymatic degradation for decolourization (Grassi *et al.*, 2011; Tan *et al.*, 2013) and performs better than fungi in low pH environments, offering greater stability in enzymatic degradation. Algae, widely found in the environment, are gaining recognition for their involvement in degrading textile dyes. They can effectively treat large volumes of dye wastewater due to their high biomass content and ability to undergo biodegradation. Algae degrade dyes through mechanisms: a) using dyes for growth, b) transforming dyes into intermediates.

Azo-dye decolourization occurs under aerobic, anaerobic, and facultative anaerobic conditions through various bacteria. Anaerobic bacteria excel at colour removal by producing azo reductases that cleave azo bonds, resulting in mutagenic aromatic amines. In contrast, aerobic activated sludge processes do not significantly contribute to colour removal. Research has explored the role of different bacterial groups in azo dye decolourization, with bacterial methods being effective due to higher biodegradation rates, low costs, less sludge production, and environmental friendliness. Bacterial degradation can utilize both pure and mixed cultures and is stable across varying pH levels

2. MATERIAL AND METHODS

Collection of soil Sample: The soil sample was collected in a clean collection bottle from the textile waste disposal site Pune, Maharashtra, Pune industry. The samples were transferred immediately to the laboratory for further analysis. Direct red dye is used in this study. The solution of the dye was prepared by dissolving 2 mg of dye in 100 mL of distilled water.

Enrichment and isolation of dye-decolorizing bacterial isolates:

Bacterial strains were isolated from the soil samples by following steps serial dilution was done, then using pour plate technique colonies were obtained and then by selecting single colony it was further streaked on sterile nutrient agar plates using streak plate technique the plates were incubated at 27°C for 24 hours. The colonies were further sub cultured a few times to obtain a pure culture. Representative well-isolated colonies were presented on nutrient streak plate at 4°C. The

isolates were analyzed for dye decolorization by point inoculating on mineral salt basal medium plus 2 mg % direct red dye and incubated at 30 °C for 72 h. The colony shows a zone of decolourization around. It was taken as a dye-decolorizing isolate. The isolate showing maximum selection ratio (S/R) was taken as the best promising isolate for decolorization of direct red dye. Out of five isolates, one was found promising and was subjected to morphological and biochemical identification. (G Zheng *et al.*, 1999)

Morphological and biochemical characterization:

Colony identification based on Gram staining, motility, and citrate utilization tests, Catalase and Oxidase test, Fermentation of sugars, IMViC tests, etc were performed for identification of potential isolates of bacteria. (T. Ahmad *et al.*, 2015)

Effect of pH on Decolourization of Direct red dye:

To investigate the impact of pH, prepared four conical flasks, where each contain 100 mL of nutrient broth, and adjusted their pH to 4, 8, 10, and 12, respectively. Inoculated each flask with a loop full of culture and incubated them at 37°C for 24 h. Following the incubation, aseptically added 2 mg of direct red dye to each flask, then returned them to the incubator and monitor for decolourization at specified time intervals. At each interval, collected samples, centrifuged them, and measured the absorbance of the supernatant at 520 nm using a UV spectrophotometer. (T. Ahmad *et al.*, 2015)

Effect of temperature on Decolourization of Direct red dye:

Prepared four conical flasks with 100mL of nutrient broth. Inoculated each flask with a loop full of culture and incubated them at different temperatures: Room temperature, 28°C, 35°C, and 40°C, For 24 h. Following incubation, aseptically added 2 mg of direct red dye to all flasks, then observed for decolourization at various time intervals. Like with the pH experiment, collected samples, centrifuge, and measure the absorbance reading of the supernatant at 520 nm by using a UV spectrophotometer to analyze the effects of temperature on the decolourization process. (T. Ahmad *et al.*, 2015)

Effect of Static and shaking conditions on Decolourization of Direct red dye:

Prepared two conical flasks, where each contain 100 mL nutrient broth. Inoculated a loop full of culture and placed in an incubator at 37°C for 24 h. After 24 h added 2mg of direct red dye in both the flasks aseptically. Kept one flask in the incubator and the other on the rotatory shaker. The decolourization after a 8 h ,16 h ,24 h, 32 h time intervals. Collected the samples at each time interval, centrifuged and using the supernatant taken the absorbance at 520 nm using a UV Spectrophotometer (Dawkar *et al.*, 2009).

Effect of metal ions on Decolourization of Direct red dye:

Prepared 3 conical flasks each of 100mL nutrient broth containing iron, lead, and calcium salts 1 mM concentration of each respectively. Inoculated a loop full of culture and placed in an incubator at 37°C for 24 h. After 24 h added 2mg of direct red dye in all the flasks aseptically. Kept the flasks in the incubator and observed for decolourization after an 8 h, 16 h, 24 h, and 32 h time intervals. Collected the samples at each time interval, centrifuge, and using the supernatant taken the absorbance at 520 nm using a UV Spectrophotometer. (Dawkar *et al.*, 2009).

Decolourization measurement:

$$\% \text{ Decolourization} = \frac{(\text{Initial O.D.} - \text{Final O.D.})}{\text{Initial O.D.}} \times 100$$

3. RESULTS AND DISCUSSION



Photoplate 1: Enrichment for dye decolourizing bacteria from soil sample at 30°C for a week.

Isolation of dye-degrading bacteria:



Photoplate:2 Growth of dye-degrading bacteria on nutrient agar medium at 30°C for 72 h.

Morphological characterization:



Image 1: Gram staining of *Bacillus* isolate (Gram positive rods with spores inside the cells).

Table: 1 Morphological and biochemical characterization of bacterial isolate.

Test	Result
Gram staining, Morphology, and sporulation	Gram positive, rods, sporulating
Motility test	Motile
Catalase test	+
Oxidase test	+
Indole test	-
Methyl Red indicator test	-
Voges-Proskauer test	-
Citrate utilization assay	+
Sugar fermentation- Glucose	+

Maltose	-
Lactose	-
Sucrose	-

Table 1: Characterization based on morphology and biochemistry (+ ➡ Test positive, - ➡ Test negative)

Under a microscope, gram-positive bacteria appear thick rod shaped with motile nature and its biochemical tests showed that it belongs to aerobic *Bacillus* spp.

Effect of pH on dye decolourization: Four flasks with 4, 8, 10, and 12 pH respectively were observed for the decolourization and biodegradation at different time intervals. Of the four pH, pH 4 showed the least dye degradation 38.46 %, and pH 8 showed the most 90.76%.

Effect of pH on dye decolourization

Time of incubation at 30°C (h)	pH 4 (Abs at 520nm)	pH 4 (%)	pH 8 (Abs at 520nm)	pH 8 (%)	pH 10 (Abs at 520nm)	pH 10 (%)	pH 12 (Abs at 520nm)	pH 12 (%)
0	0.65	0	0.65	0	0.65	0	0.65	0
8	0.48	26.15	0.234	64	0.48	26.15	0.45	30.76
16	0.45	30.76	0.225	65.38	0.45	30.76	0.414	36.31
24	0.414	36.30	0.21	67.69	0.414	36.30	0.4	38.45
32	0.4	38.46	0.06	90.76	0.4	38.46	0.48	26.15

Table: 2 Effect of pH on dye decolourization

Effect of temperature on dye decolourization: Four flasks were kept at different temperatures for growth, decolourization, and biodegradation purposes. The temperatures were- RT, 28°C, 35°C and 40°C. Among the four different temperatures, the flask kept at 28°C showed the most dye degradation was 77.69 % whereas the flask at 40°C showed the least degradation was 66.16 %.Effect of temperature on dye decolourization

Time of incubation at 30°C (h)	RT (Abs at 520nm)	RT (%)	28°C (Abs at 520nm)	28°C (%)	35°C (Abs at 520nm)	35°C (%)	40°C (Abs at 520nm)	40°C (%)
0	0.65	0	0.642	0	0.654	0	0.65	0
8	0.23	64.00	0.22	65.53	0.349	46.30	0.45	30.76
16	0.22	65.38	0.205	68.46	0.279	57.07	0.3	53.84
24	0.21	67.69	0.199	69.32	0.254	60.92	0.50	23.07

32	0.18	71.23	0.145	77.69	0.209	67.84	0.22	66.16
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Table: 3 Effect of temperature on dye decolourization

Effect of static and shaking conditions on dye decolourization: Static and shaking conditions were compared for the dye degradation. In static and shaking conditions, it is observed that the static condition shows comparatively more degradation was 76.30 %.

Time of incubation at 30°C (h)	Static Condition (Abs at 520nm)	Static Condition (%)	Shaking Condition (Abs at 520nm)	Shaking Condition (%)
0	0.65	0	0.64	0
8	0.32	49.38	0.312	52.00
16	0.33	47.84	0.25	61.53
24	0.26	58.76	0.22	64.76
32	0.15	76.30	0.27	57.07

Table: 4 Effect of static and shaking conditions on dye decolourization

Effect of metal ions on dye decolourization: The result of metal ions (Lead, Calcium, and Iron) on the dye degradation rate was measured in a couple of time intervals. The flask that contained Calcium ions showed more dye degradation activity 81.53 % than the other two flasks containing Iron and Lead respectively.

Effect of metal ions on dye decolourization

Time of incubation at 30°C (h)	Lead (Abs at 520nm)	Lead (%)	Calcium (Abs at 520nm)	Calcium (%)	Iron (Abs at 520nm)	Iron (%)
0	0.65	0	0.64	0	0.65	0
8	0.5	23.07	0.34	47.68	0.48	26.15
16	0.45	30.76	0.213	67.79	0.45	29.76
24	0.3	53.84	0.19	70.76	0.41	36.8
32	0.22	66.18	0.12	81.53	0.34	47.69

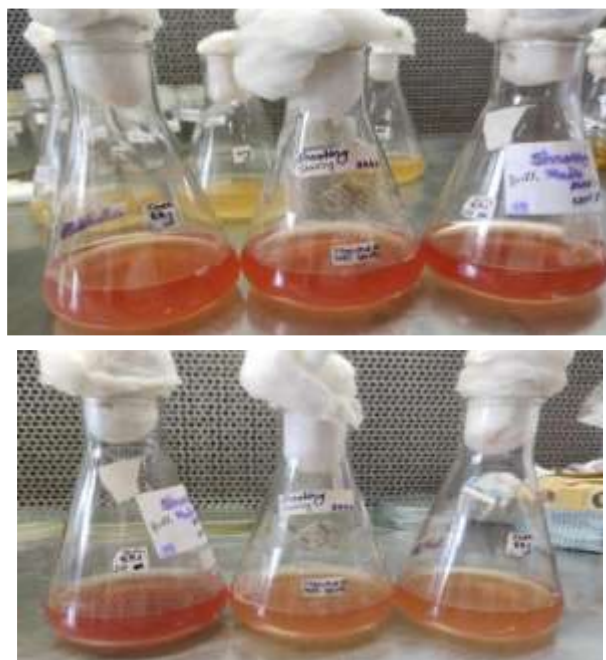
Table: 5 Effect of metal ions on dye decolourization.



Photoplate 3: Decolourization and biodegradation through the impact of pH.



Photoplate 4: Decolourization and biodegradation through the impact of temperature.



Photoplate 5: Decolourization and biodegradation using static and shaking conditions.



Photoplate 6: Decolourization and biodegradation through the impact of metal ions.

4. DISCUSSION

The Decolourization of Direct Red textile dye using *Bacillus* species has become a promising area of study. *Bacillus* species, known for their adaptability, are gram-positive, rod-shaped with spores inside the cells bacteria that thrive in various environmental conditions. They have been identified as effective agents in breaking down a range of organic substances, including textile dyes. A number of research studies have examined the decolourization potential of different *Bacillus* strains in the treatment of Direct Red dye. A similar study illustrated that the *Bacillus cereus* strain was able to remove up to 70 to 90% of the dye within 32 h, highlighting its potential in treating wastewater contaminated by textile dyes. Additionally, another study focused on the *Bacillus subtilis* strain, which showed a decolourization efficiency of up to 92 % within 72 hours. This strain's ability to function under both aerobic and anaerobic conditions makes it versatile for various wastewater treatment systems. The decolourization of Direct Red dye by *Bacillus subtilis* demonstrates the flexibility of using *Bacillus* species in diverse environmental settings. The breakdown of Direct Red dye by *Bacillus* species involves complex metabolic pathways, including the action of extracellular enzymes like laccases, peroxidases, and azoreductases. These enzymes break down the dye's chemical structure into simpler compounds that bacteria can utilize for energy and carbon. As Compared to conventional chemical treatments, biodegradation using *Bacillus* species is more cost-effective, eco-friendly, and scalable for industrial applications. However, various factors can influence the process such as dye concentration, pH levels, temperature, and the presence of other organic or inorganic substances. Therefore, careful selection of bacterial strains and optimization of environmental conditions is key to maximizing biodegradation efficiency and speed.

5. CONCLUSION

This study highlights the promising potential of *Bacillus* species in the effective decolourization of direct red dye, offering an eco-friendly and cost-effective approach to dye removal. It was found that the degradation products were less toxic compared to the original dye. Indicating the viability of this method for reducing environmental and health hazards associated with textile dyes. Additionally, the observed connection between enzyme activity and dye decolourization further emphasizes the complexity of the biodegradation process. Further research should concentrate on optimizing conditions for enhanced degradation efficiency, exploring the application of *Bacillus* species to other toxic dyes, and scaling up the process for industrial use. Ultimately, this technique holds great potential for advancing sustainable wastewater treatment technologies in future.

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