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Biodecolorization of Reactive Red Using *Bacillus cereus* Isolated from Oil Contaminated Soil

Balakumaran Manickam Dakshinamoorthi ^a, Swetha Jothiraman ^a, Santhi Rasappan ^b, Suresh Subramaniyam ^c and Jagadeeswari Sridharan ^{d*}

 ^a Post Graduate Department of Biotechnology, Dwaraka Doss Goverdhan Doss Vaishnav College (Autonomous), Chennai-600106, Tamil Nadu, India.
 ^b Tagore College of Arts and Science, Chennai-600044, Tamil Nadu, India.
 ^c Department of Biotechnology, Faculty of Science and Humanities, SRM Institute of Science and Technology, Ramapuram, Chennai 600089, Tamil Nadu, India.
 ^d PG and Research Department of Microbiology, Dwaraka Doss Goverdhan Doss Vaishnav College (Autonomous), Chennai 600 106, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Manufacturing and using organic dyes produce a variety of hazardous organic compounds which can be harmful to living organisms. Azol dyes, one of the most important synthetic organic components, are widely used in a variety of industries, particularly in the textile industry. As azo

*Corresponding author: Email: jaga.dgvc@yahoo.in;

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dyes breakdown, aromatic amines produce mutagenic products that can cause cancer in humans and aquatic life. In order to protect the environment, it was imperative to find a biological method for degrading these dyes. In recent years, microbial biodegradation has emerged as a promising method. Different bacterial isolates from oil-contaminated soil were investigated for their ability to decolorize reactive red. Under aerobic conditions at pH 7.0 and 35 °C with 0.1 mg/mL reactive red dye concentration, the isolated potential strain showed maximum decolorization of 84 % within 24 hr. Based on the 16S rRNA gene sequence analysis, the isolate was identified as *Bacillus cereus* strain BIOS MD2. A variety of agricultural wastes were supplemented along with medium to reduce the costs of the process, and extracts of apple and chikoo peels showed better decolorization rates. UV-visible spectroscopy was used to analyze the biodegradation property of reactive red, which confirmed that *Bacillus cereus* successfully degraded reactive red. The present study provides a valuable insight into practical applications for the removal of textile dye from the environment.

Keywords: Azo dyes; bacillus cereus; biodecolorization; reactive red; textile dyes.

1. INTRODUCTION

Due to rapid increase in industrialization and urbanization, several chemicals including various forms of dyes have been utilized in different industries leading to one of the major water pollutants. The discharge of various forms of dyes into the environment has created a major impact in case of aquatic creatures by preventing the penetration of light into the water causing serious consequence among the aquatic animals. Similarly, different forms of textile dyes due to their toxic nature causes adverse effects in various organs such as reproductive, nervous, kidney, liver among the human beings (Zhou et al. 2019, Ehrampoush et al. 2011, Ardila-Leal et al. 2021). Among different types of dyes, triphenylmethane, heterocyclic, azo dyes and polymeric dves are widely used and accounts for around 70% in total production of dyes (Saratale et al. 2011. Lellis et al. 2019).

The synthetic dyes are way more photolytically and chemically stable and also highly persistent in the environment compared to the natural dyes (Ramalho et al. 2004 Wang et al. 2009). The disposal of these synthetic dyes into the natural environment without anv treatment or pretreatment may cause a series of health and environmental damages (Singh and Arora 2011). It may lead to the reduction of photosynthetic activities by reducing the penetration of light into the deeper layers of water (Daneshvar et al. 2007). The reduce in photosynthetic activities would lead to the decrease in the dissolved oxygen (DO) content causing the death of organisms in the aquatic system (Chen et al. 2003).

Due to these hazards, researchers were focusing to develop a technique to lessen the impact of

dye containing wastewater on the aquatic In environment. addition to adsorption. photocatalvtic coagulation-flocculation, ozonation, and inorganic catalysis, many chemical and physical techniques have been used to decolorize dyes. These conventional processes pass many industrial dyes through, but because of their high resistance to heat, light, and oxidizing agents, they remain in the environment for a very long time (Routoula et al. 2020).

Another disadvantage of the conventional techniques used for dye decolorization is its high cost and difficulty in the technical and operational approaches which also produces large volumes of toxic products and sludge (Borchert and Libra 2001, Srinivasan and Viraraghavan 2010). Hence, biological dye-decolorization have become a promising technology for the treatment of dye containing wastewater.

The development of environmentally friendly, affordable, and advanced wastewater treatment technologies remains vital if natural resources are to be preserved and biological dyedecolorization emerges as a promising treatment method for dve-containing wastewater. Compared to various other conventional technique it produces less sludge, eco-friendly and found to be cost-efficient (Dave et al. 2015, Holkar et al. 2016).

A number of microorganisms from a variety of taxonomic groups, such as bacteria, yeast, and fungi, are capable of decolorizing colours through adsorption, biotransformation, or degradation (Ngo and Tischler 2022). A variety of enzymes are employed by microorganisms to remove dyes, particularly those produced by bacteria. These enzymes include azo reductase, DCIP- reductase, and laccase, which contribute to biodegradation inside and outside the cells, with the presence of bacteria breaking down azo dyes in anaerobic environments as well (Qiu et al. 2022). The bacterial strains which have the ability to decolorize the dyes with industrial applications are predominantly isolated from soil and water bodies contaminated with the sludges from textile effluents, petrochemical industries and sewage treatment industries (Sarayu and Sandhya 2010).

The present study was carried out to identify potential bacterial strains which are capable of decolourizing azo dye in aerobic conditions. Additionally, various parameters such as pH, temperature, dye concentration, as well as the effect of the decolourized product were also studied to understand the biological decolourization of textile dyes.

2. MATERIALS AND METHODS

Dyes and chemicals: In the present study azo dye, reactive red was procured from the local manufacturers near Tiruppur, Tamil Nadu, India. The stock solution of 1 g/L was prepared and subsequently sterilized by filtration through a 0.22 µm filter prior use. The microbiological media was procured from Himedia Pvt Ltd., India and other analytical chemicals used in the study were procured from Merck India Pvt Ltd, India.

Sample collection and processing: In the present study, soil samples were collected from different areas Choolai, Chennai, Tamil Nadu, India which are contaminated by textile effluents and were stored at 4 °C for further use. The samples were serially diluted, plated on nutrient agar and incubated for 24 hr at 37 °C. After 24 hr, colonies with distinct morphology were chosen, maintained in nutrient agar medium and the isolated pure culture strains were used for screening for dye decolourization.

Screening of reactive red decolorizing organisms: For primary screening of the reactive red decolourization, the isolated pure culture strains were streaked on nutrient agar amended with reactive red (0.1 mg/mL) and the plates were incubated for 24 hr at 37 °C. After 24 hr, the strains which showed zone of clearance around the colonies were selected and utilized for further study.

Decolorization experiment: For broth decolourization assay, Luria Bertani broth

incorporated with reactive red (0.1 mg/ml) was prepared and the strains which showed zone of clearance in the plate screening method were inoculated. The inoculated medium was kept for incubation at 37°C for 24 hr. After 24 hr, the supernatant was collected and the decolorization activity of the strains were analysed using UV-Vis spectrophotometer at 518 nm (Pandey et al. 2016). Briefly, after 24 hr of incubation, the supernatant was collected by centrifuging the culture suspension at 10,000 rpm for 10 mins at 4 °C. The sterile nutrient broth was used as a blank and nutrient broth with reactive red (0.1 mg/mL) at 0 hr was considered as initial dye concentration.

The decolourization percentage of the isolated strains was calculated using the formula reported earlier.

Percentage of decolourization = (Initial absorbance – Final absorbance/ Initial absorbance x 100)

The strain which showed maximum decolourisation in the broth experiment was considered as potential strain and utilized for further studies.

Optimization decolorization of process: Several physico-chemical parameters such as pH, temperature, incubation time, initial dye concentration and different carbon sources related to dye decolourization by the potential isolate were optimized (Pandey et al. 2016). The potential strain was tested for its ability to decolourize reactive red dye. In this study, we investigated the effects of one parameter at a time while keeping the others unchanged. The efficiencv decolorization of the potential strain was analysed by incubating the broth at different time intervals (at every 6 hr interval), varying the pH (6.0, 6.5, 7.0, 7.5 and 8.0), temperature (25 °C, 30 °C, 35 °C and 40 °C), initial dye concentration (0.1 mg/mL to 1 mg/mL) and carbon source (1% - Glucose, Lactose, Maltose, Mannitol, Xylose and Sucrose).

Identification of potential dye decolorizing strain: The potential bacterial strain which showed maximum decolourization was identified using 16S rRNA partial gene sequencing as reported earlier (Agrawal et al. 2014). The genomic DNA of the bacterial strain was extracted, and the 16S rRNA gene was amplified using PCR by two universal primers F-5'ACG CGT CGA CAG AGT TTG ATC CTG GCT-3' and R-5'GGA CTA CCA GGG TAT CTA AT-3'. The PCR products were further purified and subjected for sequencing using dye terminator kit. The 16S rRNA gene sequence was then compared with the related sequence from different bacterial species, retrieved from the GenBank database using BLAST algorithm and was identified. Further, the sequence was deposited in GenBank and the accession number was obtained (El-Rakaiby et al. 2013).

Dye decolorization using agricultural wastes: Agricultural wastes including custard apples (Annona squamosa L.), chikoo (Manilkara zapota (L.) P. Roven) and apple (Malus domestica L Borkh) peels were collected, dried and powdered. One gram of powdered fruit peels was added to 100 mL of distilled water and kept in a rotary shaker incubator for 48 hr before the extract was filtered through Whatman No. 1 filter paper (Sameer et al. 2017, BalaKumaran et al. 2015). As part of the decolorization experiment, the fruit extract was mixed with nutrient broth with a concentration of (10 to 50 % of fruit waste extract) and sterilized. A sterilized substrate was seeded with 1% of inoculum and incubated at 35 °C for 24 hr. After the experiment, the decolorization percentage was determined spectrophotometrically at 518 nm.

Biodegradation studies of reactive red: The preliminary degradation of reactive red in the decolourized broth was analysed using UV-Visible spectroscopic studies. Briefly, the 24 hr decolourized broth was centrifuged at 10,000 rpm for 10 mins at 4 °C and was subjected to UV-Visible spectrum studies between 200 and 800 nm. Nutrient broth with reactive red and distilled water with reactive red also subjected to UV-Visible spectrum analysis for comparison (Kalyani et al. 2009).

Statistical analysis: The experiments were conducted in triplicate and data are presented as means \pm standard deviation.

3. RESULTS AND DISCUSSION

In this study, 16 different bacterial strains with distinct colony morphology were isolated from 9 samples. In the preliminary plate screening assay, 6 isolates found to have decolorization property which was observed based on the zone of clearance around the colony. These strains were selected and subjected for their reactive red decolourization ability using broth assay (Fig. 1).

Among the six strains, one potential strain showed a maximum decolourization of 72.47 % at 24 hr, while the other strains show lesser. Hence the potential strain was used for further studies.



Fig. 1. Reactive red decolorization. a) Nutrient broth with reactive red before incubation; b) Decolourization of reactive red in nutrient broth after 24 hr of incubation

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Fig. 2. Effect of incubation time on reactive red decolorization



Fig. 3. Effect of initial medium pH on reactive red decolorization



Fig. 4. Effect of initial dye concentration on reactive red decolorization

We investigated the decolourization ability of potential strains at different time intervals. The decolourization of reactive red increases with increasing incubation time from 12 to 24 hr. Further, the decolourization percentage did not increase after 24 hr, even less variation was observed between 18 and 24 hr, so 18 hr of incubation was found to be an optimal time for further studies (Fig. 2). This was due to the actively growing state of bacterial strains which tend to produce various enzymes leads to the effective decolourization (Pandey et al. 2019). Sadeghi and coworkers (2019) have demonstrated that bacterial strains isolated from dairy effluent can decolourize reactive red 120. They also discovered that incubation time increases decolourization by up to 96.1% at 24 hr. Similar results have been reported previously by various researchers which is well correlated with our study (Kale et al. 2010, Srivastava et al. 2022, Sennaj et al. 2023).

The effect of initial pH of the medium on dye decolourisation was studied and optimized. pH ranging from 6.0 to 8.0 was studied and maximum of 79.64 % was achieved when the initial pH was maintained at 7.0, while 72.84 % and 64.87% decolorization was achieved when the initial pH of medium was at pH 7.5 and 6.5, respectively (Fig. 3). The decolourization reactive red dye found to be very low when the pH was maintained below 6.5 and above 8.0. Similar to our results, Pandey and his coworkers (Pandey et al. 2019) investigated the effect of different pH in the medium to assess the decolourization efficiency of the potential isolate in the MSM medium. They found that the maximum decolourization was observed when the pH was maintained at 7.5 with 87.47%. The effective decolourization may be due to the production of azo reductase enzyme during the process, and they also reported that the production of enzyme varies depending up on the medium pH.

Various reported that studies have the decolorization efficiency of a textile dye is greatly affected by the initial dye concentration in the given environment (Pathak et al. 2014, Singh et al. 2015). In the present study, different concentrations of reactive red dye (0.1 mg/mL to 1 mg/mL) were used to examine the ability of bacteria to decolorize. As seen in the Fig. 4, increasing the initial dye concentration in the medium decreases the decolourization efficiency of the potential isolate. At the end of 18 hr of incubation. the maximum decolorization efficiency of the strain was found to be 84.56 %

when the concentration of dve was maintained at 0.1 mg/mL (Fig 4). The decolourization found to be decreasing drastically when the concentration of the dve was increased form 0.2 mg/mL to 1 mg/mL which may be due to the toxic effects of textile dye against the isolate thus ceasing their active growth. Researchers have performed similar studies and reported that increasing dye causes decolorization concentrations to decrease (Jadhav et al. 2008, Pandey et al. 2019). According to our findings, the initial dye concentration significantly affects the decolorization process. With an increase in dve concentration, decolorization rates decrease, factors may contribute to Several this phenomenon, including the natural toxicity of dye concentrations, which might inhibit microbial activity in most organisms. The findings of our study are in agreement with those of previous studies that have shown a decreased decolorization rate with an increase in dye concentrations (Lakshmipathy et al. 2010, Al-Tohamy et al. 2020). An azo dye reaction orange 16 was decolorized by Marine Nocardiopsis sp. at a concentration of 50 mg/L with a 95% decolorization rate (Chittal et al. 2019). In another study, Streptomyces DJP15 was examined for its ability to decolourize dye at various concentrations. With an initial dye 50 concentration of mg/L, maximum decolourization was observed at 76.66% (Pillai 2017).

The incubation temperature plays significant role on the decolourization efficiency of various textile dyes as it is directly having an impact of the microbial growth in the given condition (Meerbergen et al. 2018, Hossen et al. 2019, Kameche et al. 2022). In the present study, the maximum decolourization was observed when the incubation temperature was maintained at 35 °C showing 81.82%. When the temperature is below 25 °C, the decolorization efficiency is reduced (Fig. 5). Decolorization efficiency decreased as the temperature increased above 35°C, due to decrease in cell viability.

Our findings are also in accordance with these reports, where maximum dye decolourisation was observed when *S. albidoflavus* 3MGH was incubated at 35°C showing against azo dyes (El Awady et al. 2024). A similar finding was reported by Khan and Malik (2018) who tested *Arthrobacter soli* BS5 for its ability to decolorize reactive black. When incubated at mesophilic temperatures, the strain showed increased rates of decolorization.

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Fig. 5. Effect of incubation temperature on reactive red decolorization



Fig. 6. Effect of different carbon source on reactive red decolorization

The ability of Bacillus cereus on decolorization efficiency of reactive red in the presence of various carbon sources was also studied (Fig. 6). Of the different carbon sources tested, glucose was the optimal carbon source, showing maximum decolorization of reactive red with a maximum dye decolorization of 88.26 %, followed by sucrose with 76.74% and lactose 73.51%. Researchers studied with the decolorization of azo dyes with Streptomyces albidoflavus 3MGH in a medium amended with various carbon sources (El Awady et al. 2024). A higher level of degradation was observed when sucrose was used as a carbon source. Providing

nutrients to microbial growth and metabolic activities is crucial for biodegradation. By altering enzyme activities and metabolic pathways, different carbon sources can affect the efficiency of dye decolorization (Saratale et al. 2009, Przystaś et al. 2018, Salem et al. 2019).

Based on a 16S rRNA gene sequence, the potential strain was identified as *Bacillus cereus*. According to GenBank's database, *Bacillus cereus* was the closest match with 99% sequence identity. The sequence information was submitted to Genbank under accession number MH145410.1.



Fig. 7. Biodegradation of reactive red studies using UV-Visible spectroscopy. Absorption spectra of reactive red a) in distilled water, b) in nutrient broth, c) in decolourized broth



Fig. 8. Utilization of various agro-industrial wastes for reactive red decolourization studies.

In this study, spectroscopic measurements were carried out on the decolorized supernatant for UV-visible spectrum ranges between 200 and 800 nm to analyze preliminary degradation of reactive red. According to the results, the absorption peak was maximum observed between 520 and 540 in the nutrient broth supplemented with reactive red, but not after decolourization after optimal incubation (Fig. 7). The absence of peaks in the decolorized broth confirms Bacillus cereus ability to degrade reactive red. Several other studies have also confirmed similar results of dve degradation using UV-visible spectroscopy studies (Akansha et al. 2022, Yadav and Singh 2024).

A variety of agricultural wastes including custard apples, chikoo, and apple peel extracts were investigated as medium sources to decolorize reactive red using *Bacillus cereus* in the present study (Fig. 8). Concentrations ranging from 10 to 50 % of the extracts were added along with medium and their decolourisation potentials were determined.

When custard apple peel extract (30% in the medium) was used, maximum decolourisation was observed at 68.27 %. Supplementing the medium with 20% and 30% of apple and chikoo peel extracts resulted in maximum decolorization of 42.88 % and 41.11 %, respectively. Researchers have also assessed decolourization potential using medium supplemented with various agricultural wastes (Eltarahony et al. 2021, Unuofin 2020, Almeida et al. 2018). Agroindustrial wastes, which are a great source of readily available carbon, can be used as substrates and supporting agents for decolourization of various dyes using bacterial strains (Maniyam and Hari 2021).

4. CONCLUSION

We have isolated a dye-degrading strain from oilcontaminated soil and identified it as Bacillus cereus by 16s rRNA gene sequencing. The strain was more effective at decolorizing dyes. Several physiological and chemical factors were examined for maximum decolorization at the laboratory scale. Different carbon sources and agro-industrial wastes were incorporated into the medium and investigated for decolourization. The strain's ability to decolorize dve under different conditions made it a potential candidate for wastewater treatment in textile industries. According to the overall results, the strain *Bacillus cereus* is capable of decolorizing azo dyes and can also be used to treat wastewater. However, more research is needed to determine if the decolorized product is toxic and whether it can be used for large-scale processes.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative Al technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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