RAPID REMOVAL OF AN AZO DYE USING A BACTERIAL CELL ISOLATED FROM THE INDIGENOUS POPULATION PRESENT IN INDUSTRIAL EFFLUENT

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

By

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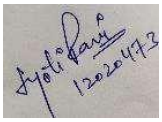
LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2025

DECLARATION

I, hereby declared that the presented work in the thesis entitled "Rapid Removal of an Azo Dye Using a Bacterial Cell Isolated from Indigenous Population Present in Industrial Effluent" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Umesh Gautam, working as Head of Department, in the Department of Molecular Biology and Genetic Engineering of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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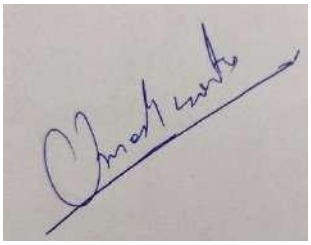
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Rapid Removal of an Azo Dye Using a Bacterial Cell Isolated from Indigenous Population Present in Industrial Effluent" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Microbiology / School of Bioengineering and Biosciences, is a research work carried out by Jyoti Rani, 12020473, is Bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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Dedicated

To

My Beloved Parents and My cherished

Husband

ABSTRACT

Abstract

AZO dyes, named after the azo functional group (-N=N-) that is a defining characteristic of this class of colorants, have been widely used in various industries since their development in the mid-19th century. These synthetic dyes are particularly prominent in the textile industry, where they are applied to fabrics to impart vibrant and long-lasting hues. The versatility of AZO dyes has led to their extensive application in diverse sectors, including paper printing, cosmetics, and even pharmaceuticals.

The presence of synthetic dyes, such as methyl orange, in wastewater effluents has become a significant environmental concern due to their persistent and potentially toxic nature.

Bioremediation, using microorganisms to degrade or remove hazardous substances, presents a promising approach to address this environmental issue. This study focuses on exploring the potential of a novel bacterial strain to effectively decolorize and biodegrade methyl orange, a widely used and persistent azo dye that is often found in wastewater effluents. Eleven bacterial strains were isolated from industrial wastewater samples collected in the city of Panipat, India. Among this diverse collection of microorganisms, out of eleven bacterial strains, the isolated Bacillus cereus J4 was assessed for its potential for bioremediation. Bacillus cereus J4 significantly reduced the amount of methyl orange (MO). This work has examined the removal of MO in the liquid phase.

To further optimize and fine-tune this bioremediation process, two complementary experimental design approaches - the Plackett-Burman method and the Response Surface Methodology. The Plackett-Burman design allowed us to identify the significant factors influencing the removal efficiency, while the more sophisticated Response Surface Methodology provided a comprehensive optimization by accounting for the complex interactions between these various parameters.

The Plackett-Burman experimental design approach allowed to systematically identify the significant factors that had a significant impact on the removal efficiency of the Methyl Orange dye. This initial screening step provided critical insights into the critical parameters that needed to be optimized further. Building on these findings, the more

advanced Response Surface Methodology, offered a more comprehensive optimization by accounting for the complex interactions among the influential factors.

Plackett-Burman (PB) and Central Composite Design (CCD), respectively, tested and refined critical operating parameters in batch research that took removal efficacy into account as a response. The initial pH, temperature, initial dye concentration, and additional nitrogen source of the system were found to be significant factors in the removal of MO. At an initial pH of 7.0, initial temperature of 37 ^{0}C, initial concentration of 50 mg/l, and nitrogen source of 2% (w/v), around 98.05% removal of MO was accomplished within 48 hours. Possible relationships between Fourier Transform Infrared Spectroscopy (FT-IR) and Field Emission Scanning Electron Microscopy (FE-SEM) images provided insights into the structural alterations that occurred due to the interaction with Methyl Orange dye.

A thorough continuous column study was conducted using whole-cell immobilized alginate beads as the column packing material to assess the potential for large-scale removal of methyl orange (MO). The study demonstrated an impressive removal of Methyl Orange (MO) at a continuous flow rate of 0.5 and 1.5ml/min. Additionally, an in-depth evaluation of phytotoxicity and microbial toxicity revealed that MO treated with Bacillus cereus J4 showed significantly lower toxicity levels compared to its native form, particularly in terms of environmental and agricultural impact. This comprehensive investigation showcased the bacterium's remarkable capability for dye removal, positioning it as a promising and efficient resource for future bioremediation efforts.

In summary, the research findings highlight the potential for using a bacterial strain to treat dye-contaminated wastewater, offering an eco-friendly and cost-effective solution to this environmental challenge. The bacterium *Bacillus cereus* J4 showed significant promise in effectively removing the sulfonated azo dye Methyl Orange under optimal conditions identified in this study, with the ability to remove up to 98% of the dye within 48 hours. This demonstrates its potential for practical application in treating textile effluents contaminated with similar synthetic dyes. To further develop this bioremediation approach, future studies should explore scaling up the process to assess its viability for real-world industrial settings. Additionally, it would be valuable to investigate the strain's effectiveness in remediating a broader range of commonly used

industrial dyes to develop a comprehensive, broad-spectrum bioremediation strategy to address the environmental challenges associated with discharging synthetic dyes into aquatic ecosystems.

Keywords: Azo Dye, Bioremediation, Bacterial Strain, Decolorization Isolation, Immobilization, Methyl Orange, Optimization, RSM, and Toxicity,

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Strength doesn't come from what you can do, it comes from overcoming the things you once thought you couldn't

First of all, I am grateful to the almighty god (Shri Krishna) for blessing me to complete my research work peacefully and successfully. It was my father's dream to do a doctorate and I had tried my best in everything to achieve this goal and fulfill his dream for me by her uncountable blessings.

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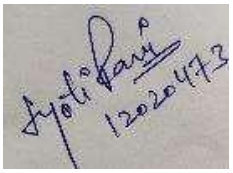
Mummy and Papa, thanks for your constant support and enthusiasm. I dedicate this dissertation to my parents as an expression of my appreciation and love. The time I have spent here at Lovely Professional University in pursuit of my doctorate will be unforgettable throughout my life.

I would like to express my heartiest appreciation to all those who have helped make my time here a great experience.



Jyoti Rani

PREFACE

Panipat known as the big textile hub of Haryana, is well-known for dyeing and printing of fabric. Day by day, an increase in the usage of chemical-based dyestuff and their direct or indirect release into natural surface water bodies and agricultural land is contaminating the environment and posing an ecological hazard. Considering the crucial situation, the current study was focused on assessing the contamination load of textile effluent from the textile industries located near the industrial area, near the larger drain. Also, an effort has been made to isolate and identify indigenous bacterial strains that are proficient in degrading and detoxifying the selected Azo dye Methyl Orange. The uses of microorganisms (bacteria) for the removal of dye from textile effluents will serve two purposes at the one time in terms of cost and is an environmentally-friendly approach for the bio-degradation of the textile dyes.

The selection of bacterial species was inspected to check their latent to degrade the selected dye. It was considered that the dye was a source of energy for bacteria showing decolorization. The mechanisms underlying the dye degradation by bacteria were explored. The possible role of dye-degrading enzymes and degraded products after dye degradation was examined. Statistical Optimization of selected bacteria has been developed and utilized by immobilizing them on adsorbent for removal of dyes, which provided an insight into the execution of the microbe-based methodology aimed at bioremediation of textile wastewaters from “lab to field”.

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Abbreviations:

(A^0) Initial absorbance

(AlgHG) Alginate hydrogel

(A^t) Final Absorbance

($CaCl_2$) Calcium chloride

(CCD) Central Composite Design

(ChiHG) Chitosan hydrogel

(FESEM) Field Emission Scanning Electron Micrographs

(FTIR), Fourier transformed infrared spectroscopy

(HCL) Hydrochloric Acid

(HPLC) High-performance liquid chromatography

(I) Initial concentration

(*m*)The total mass of the adsorbent, (g)

(MO) Methyl orange

(NA) Nutrient agar

(NaOH), Sodium

hydroxide (NB), Nutrient

Broth

(n_c) Number of the central run, in CCD designs

(OD), Optical density

(OVAT) One variable at a time

(PB) Plackett Burman

(RSM) Response surface methodology

(T) Temperature

(U) Urea

CHAPTER 1

INTRODUCTION

CHAPTER 1 – INTRODUCTION

Environmental pollution, including water and air pollution, presents a critical challenge to humanity and ecosystems. The slogan of the 21st century, “Nothing is holy, sacred, or off limits when everything is for sale”, clearly shows the severity of water resource mismanagement. Throughout history, human activities have significantly affected rivers and streams (Wohl, 2020). Despite there being enough water for everyone, mismanagement and corruption restrict access to this vital resource. Current environmental trends, such as pollution, population growth, and climate change, highlight the need for urgent action (Agache et al., 2022). Human exploitation of natural resources has created a new environmental realm, the anthrosphere, which impacts the Earth's systems. Preserving high-quality water resources is essential for all life forms, including humans. However, population growth and economic development have worsened pollution, with industrial and municipal waste contaminating water sources (Silva et al., 2020). Over the past few decades, industrialization has been a primary contributor to water pollution. A variety of pollutants enter water bodies, causing both aesthetic degradation and harmful effects on aquatic and human life (Chowdhary et al., 2020). The wastewater from factories, municipal amenities, and agro-industries contains a variety of contaminants, such as bacteria, pesticides, heavy metals, hormones, dye-causing microorganisms, dyes, and other inorganic and organic substances. Textile industries, for instance, are significant water consumers and discharge substantial volumes of wastewater into water bodies (Kishor et al., 2021). This continuous pollution poses serious threats to the health of ecosystems and human populations reliant on clean water sources.

Textile industries stand out as major consumers of dyes, with over 7×105 metric tons of dyes commercially available (Sudarshan et. al., 2023). The textile industry is crucial for the economies of India and China, providing jobs for millions of people and making a significant contribution to the countries' overall economic output. However, the textile industry is also associated with substantial environmental challenges, particularly in regions like Panipat, India, where it contributes to highly polluted discharge water, adversely affecting public health (Arif et al., 2021). The pollution of water bodies by textile dyes poses significant health hazards, with studies linking the textile industry

pollution to cancer diagnoses in nearby populations (Khan et al., 2023). Moreover, the contaminated water affects soil health and agricultural production due to harmful chemicals, impeding crop germination and growth. Textile industries have a disproportionate impact on the environment, consuming large volumes of water and producing enormous quantities of wastewater during processing and dyeing. It's estimated that 52 gallons of water are needed to process 1 kg of textile, with various chemicals used in de-sizing, scouring, bleaching, mercerizing, and dyeing stages (Oliveira et al., 2021). The dyeing and finishing steps, in particular, contribute the main pollutants to textile wastewater, highlighting the need for stringent environmental regulations and sustainable practices in the textile industry.

Industrial dyes can be categorized based on their sources, chemical structures, and application (Slama et. al., 2021). Chemical structure classification aids dye chemists by revealing dye families with similar properties, while application-based classification is employed by the Color Index, facilitating dye nomenclature. Natural dyes, derived from plants, animals, or minerals, offer a blend of substances that can be mordanted and categorized into vat or acidic dyes (Hamdy et al., 2021). They are limited to natural or regenerated fibers due to their lesser color brilliance compared to synthetic dyes. Disperse dyes, extensively used in the textile industry, color polyesters and cellulose acetate fibers. They are non-ionic, insoluble dyes, with azo dispersion dyes being the most commonly used (Meena et al., 2022). However, their release into the environment poses significant toxicity risks and challenges in degradation. Synthetic dyes offer greater color variation and ease of synthesis compared to natural dyes, finding widespread use in various industries like paper, tannery, and cosmetics (Mehta et al., 2021). They are classified based on the applications and chemical structures, encompassing direct dyes, acid dyes, mordant dyes, basic dyes, reactive dyes, vat dyes, sulfur dyes, azoic dyes, solvent dyes, disperse dyes, and pigments (Lord et al., 2023). Each type of synthetic dye presents unique challenges in wastewater treatment due to its chemical composition and environmental persistence.

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isolates are more effective in degrading synthetic dyes. Moreover, microorganisms have been found capable of removing toxic dyes and metals from contaminated water and soil (Tripathi et. al., 2023), (Lazer et.al., 2024). In the context of textile wastewater treatment, the use of microorganisms holds significant promise (Mishra et al., 2023). Specifically, bacteria isolated from textile effluents have shown potential in decolorizing and degrading synthetic dyes, such as dispersion dyes (Haque et. al, 2022), (Yang et. al., 2024). This approach not only addresses water pollution caused by textile industries but also offers a sustainable solution for wastewater management. However, conventional treatment methods often fall short in treating textile wastewater due to the complex nature of dye molecules. Bioremediation, on the other side, offers a viable alternative by leveraging the natural capabilities of microorganisms (Rane et al., 2021). In this study, the focus is on evaluating the potential of indigenous bacterial strains to biodegrade and decolorize azo dye Methyl Orange (MO), commonly used in textile industries. The significance of this study lies in its possible efficiency to provide cost-effective and environmentally friendly solutions for treating industrial effluent containing Methyl Orange dye. By harnessing the capabilities of indigenous bacteria, this research aims to contribute to sustainable wastewater management practices in the textile industry. Looking ahead, future research could explore the broader applications of isolated bacteria in degrading various dye categories. However, due to the complexity of dye molecules and time constraints, such studies may be challenging. Additionally, further investigations could focus on addressing other pollutants present in textile wastewater samples, which may impact bacterial decolorization processes.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2 – REVIEW OF LITERATURE

2.1 Water Quality and Public Health

Water is a limited and valuable resource on Earth, necessitating strict measures for its quality refinement and preservation. However, anthropogenic activities have significantly affected access to clean drinking water by introducing various pollutants into natural water systems (Ogidi et al., 2022). These pollutants originate from multiple sources, including oil spills, resource mining, nuclear waste leaks, agricultural practices, and industrial discharges. One of the most concerning pollutants in water resources is industrial effluents, particularly from textile, dyeing, paint, and tannery industries (Islam et al., 2023). These industries release hazardous chemicals and dyes into the environment continuously. It's been estimated that seven million tones of dye are sent (released) into water bodies annually (Zhang et al., 2012). This substantial discharge poses a severe threat to the environment by altering sunlight penetration in water, which restricts photosynthesis in aquatic flora and thereby reduces the productivity of aquatic ecosystems. The continuous release of dye-laden effluents has profound environmental and health implications. Even trace amounts of dyes (1 ppm) in water are highly visible and toxic (Rafiullah et. al., 2010; Kumar et. al, 2010). The chemical structure of dye confers resistance to fading upon light exposure, making them persistent pollutants (Chuah et al., 2005). Moreover, dyes in water can cause various health-related issues, including cancer, skin irritation, dermatitis, allergies, & genetic mutations (Etim et al., 2016). The textile and leather industries are particularly notorious for their substantial dye losses into water, estimated at up to 84,000 tonnes annually, which accounts for twenty percent of industrial waste water pollution (Kant, 2012). Dyes such as methyl orange from these industries have carcinogenic, mutagenic, and teratogenic effects on living beings (organisms), posing significant risks to humans, aquatic animals, and microorganisms.

The persistent discharge of dyes and other hazardous chemicals from industrial processes highlights the urgent need for effective wastewater treatment and stringent environmental regulations to mitigate their impact on water quality and the

public health. Addressing these issues is critical for sustaining the ecological balance and ensuring the accessibility of clean and harmless water for future generations.

2.2 Methyl Orange

MO is a synthetic pH indicator dye widely utilized in analytical chemistry for detecting changes in pH. Known for its reddish-orange appearance, methyl orange undergoes a change in color from red to yellow within a pH range of approximately 3.1 to 4.4, making it an essential tool in titrations. Beyond analytical applications, methyl orange is employed extensively in the dyeing processes of textiles and papers, and in the manufacturing of colored pigments. Its versatility extends to the production of certain medications and as a colorant in foods and cosmetics due to its vibrant coloration and stability.

2.2.1 Molecular Structure

Methyl orange is an organic compound with the chemical formula $C_{14}H_{14}N_3NaO_3S$. Its molecular structure is characterized by an azobenzene chromophore and a sulfonate group, which contribute to its unique properties. At the core of methyl orange is a central aromatic ring, specifically a benzene ring, composed of six carbon atoms and six hydrogen atoms arranged in a flat, planar structure. This benzene ring is substituted with various functional groups that enable its function as a pH indicator. The chromophore responsible for the colors change in MO is an azo group (-N=N-), consisting of 2-N atoms doubly bonded to each other. In acidic solutions, this Azo group becomes protonated, causing the molecule to appear red. Conversely, in basic solutions, the azo group is deprotonated, resulting in a yellow color. One of the nitrogen atoms in the azo group is bonded to a methyl ($-CH_3$) group. Although this methyl group does not participate directly in the color changes, it influences the solubility of MO in water. The other nitrogen atom is bonded to a sulfonate ($-SO_3Na$) group, which is negatively charged. This sulfonate group enhances the solubility of methyl orange in water and contributes to its stability at high pH levels. Additionally, the molecular structure includes two amino ($-NH_2$) groups attached to the aromatic ring. These amino groups do not directly affect the

color changes of methyl orange, but they play a role in the molecule's stability and reactivity. Overall, the combination of these functional groups in the molecular structures of methyl orange, particularly the azochromophore, methyl group, sulfonate group, and amino groups, makes it an effective and versatile pH indicator (Shang et al., 2015) (Figure 2.1).

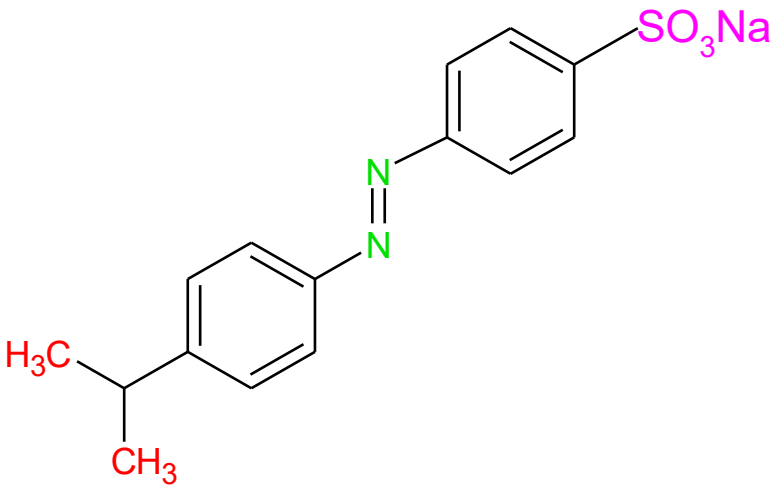


Figure 2.1: Molecular structure of methyl orange

2.2.2 Crystal Structure

Methyl orange, a small organic molecule with mol wt. of 327.4 g/ mol, exhibits a complex but characteristic arrangement of atoms. Its structure can be elucidated through several key features, despite being less defined than those of larger molecular crystals. The molecule consists of carbon, hydrogen, nitrogen, oxygen, and sulfur atoms. It features a central azo group (-N=N-) flanked by two aromatic rings. One aromatic ring bears a sulfonic acid group (-SO_3H), while the other is substituted with a methyl group (-CH_3). Additionally, a sodium cation (Na^+) is commonly associated with the sulfonic acid group, contributing to the overall structure. In the solid state, methyl orange forms various intermolecular interactions that define its crystal structure. Hydrogen bonding plays a crucial role, particularly involving the sulfonic acid group and adjacent molecules, potentially leading to the formation of molecular chains or sheets, depending on the packing arrangement. Moreover, the aromatic and methyl groups can engage in pi-stacking interactions, where overlapping electron clouds from adjacent molecules stabilize the structure. These interactions significantly influence the

molecule's optical properties, such as absorption and emission, as well as its stability and solubility in different solvents. The crystal structure of methyl orange has been investigated using X-ray crystallography, a technique essential for determining atomic and molecular arrangements in crystals. However, due to the relatively small size of the molecule, its crystal structure is not highly resolved and has not been extensively documented in the literature (Sharma and Sharma, 2013). This lack of detailed resolution presents challenges but also opportunities for further study to fully understand the intricate details of methyl orange's crystalline form.

2.3 Applications of Methyl Orange

2.3.1 pH Indicator

Methyl orange serves as a versatile pH indicator with widespread utility across various fields. It is particularly renowned for its role in acid-base titrations, undergoing a distinct color transition from red to yellow as the pH of the solution decreases from approximately 4.4 to 3.1, thus marking the endpoint of the titration process. For eg, in a study featured in the Journal of Chemical Education, methyl orange facilitated the titration of hydrochloric acid with sodium hydroxide solution. Additionally, it finds application in assessing the efficacy of water treatment methods by monitoring pH changes post-treatment, as evidenced by its usage in determining the pH of treated wastewater. Quality control processes in industry (food, cosmetics, and pharmaceuticals) also benefit from the pH testing capabilities of MO. It was also employed as a pH indicator to assess the acidity levels in shampoo and conditioner products. Moreover, methyl orange finds biological applications, including pH testing of plant tissues and animal digestive tracts, as well as in microbiology for assessing the pH of culture media used in microbial growth studies.

2.3.2 Pharmaceutical Application

Methyl orange emerges as a valuable analytical tool within the pharmaceutical sector, finding diverse applications ranging from pH determination to impurity detection and

drug release studies. Besides its role in pH determination, methyl orange demonstrates sensitivity in detecting impurities within drug substances, as illustrated in a study documented in the Journal of Pharmaceutical and Biomedical Analysis, where it discerned trace amounts of iron in pharmaceutical preparations down to concentrations as low as 0.1 ppm. Moreover, in drug release studies, methyl orange acts as a pH indicator to monitor pH variations during the release process, as evidenced by its application in a study published in the International Journal of Pharmaceutics. Furthermore, methyl orange, in conjunction with other reagents like N-bromosuccinimide (NBS) and as part of industrial quality control procedures, indigo carmine has been used in the formulation of pharmaceuticals, such as pantoprazole sodium sesquihydrate. This allows for the routine and quick identification of bulk tablets and samples. However, challenges such as its limited pH range and susceptibility to interference from other substances underscore the need for ongoing research aimed at expanding the scope of methyl orange's applications in pharmaceuticals while enhancing its sensitivity and selectivity for diverse substances.

2.3.3 Methyl Orange as a Dye

Methyl orange, a renowned azo dye, holds significant importance in various industries, including textiles and leather. In the textile sector, it serves as a vibrant dye, imparting hues of orange, yellow, and red to fabrics. This water-soluble dye can be applied through immersion dyeing, printing, or painting techniques on fabrics like cotton, wool, and silk. Its superior wash and light fastness properties render it an ideal choice for textile applications. Moreover, methyl orange functions as a pH indicator during textile processing, transitioning from red to yellow as the solution's pH becomes more basic. This color shift aids in determining the optimal pH for dyeing or printing processes, ensuring the fabric's color and durability. Research indicates that methyl orange yields bright, enduring colors in cotton and silk fabrics, as evidenced by studies that showed by Hemamalini et al(2019).

Within the leather industry, methyl orange serves as both a dye and an indicator in processing. It is utilized to dye leather, producing shades ranging from orange to yellow, employing methods like immersion dyeing and spraying. Similar to its textile counterpart, methyl orange exhibits commendable lightfastness properties, making it a

preferred choice for leather dyeing. Studies, including research by Bhanu et al. (2015), affirm methyl orange's efficacy in leather dyeing, noting its ability to generate vivid and enduring colors (Bhanu et al., 2015). In summary, methyl orange emerges as a versatile compound, finding extensive applications in both the textile and leather industries. Its role as a dye enables the production of vibrant colors in fabrics and leather, while its function as a pH indicator aids in maintaining optimal processing conditions. Research underscores methyl orange's favorable colorfastness properties, establishing its efficacy in enhancing the aesthetic and functional qualities of textiles and leather products.

2.3.4 Paper and Food Industries

Methyl orange's distinctive ability to undergo color changes in acidic and basic environments makes it invaluable in the food industry for pH measurement and control. It plays a pivotal role in ensuring optimal pH levels during the production of various food products such as sauces, pickles, and canned goods. Additionally, in dairy product manufacturing, methyl orange assists in maintaining the requisite pH for bacterial culture growth in products like cheese and yogurt. Moreover, it facilitates acidity testing of fruits and vegetables to ascertain their suitability for canning processes.

In the paper manufacturing sector, methyl orange serves as a crucial tool for monitoring and controlling pH levels throughout the production process. By testing the pH of pulp before its amalgamation with other constituents, methyl orange influences both the quality of the final paper product and the efficiency of the manufacturing process. Recent research endeavors, exemplified by studies like that by Ashok et al. (2017), focus on optimizing methyl orange concentrations in food products and developing novel detection methods in the paper industry (Ashok et al., 2017). These initiatives aim to enhance pH measurement accuracy and control, thereby elevating manufacturing process efficiency and product quality.

2.4 Environmental constraints of Methyl orange

Despite its utility, methyl orange presents significant environmental challenges due to its synthetic nature. The production process of methyl orange can release hazardous by-products and waste, including toxic intermediates like aromatic amines, contributing to

environmental pollution if not properly managed. Its chemical stability leads to prolonged persistence in soil and water bodies, where it can accumulate and disrupt ecosystems by affecting microbial communities and aquatic organisms. Methyl orange's presence in water bodies can reduce sunlight penetration, impacting photosynthetic aquatic life and leading to bioaccumulation in tissues of fishes &other organisms, thereby propagating toxic effects through the food chain. Regulatory agencies classify methyl orange as a hazardous substance because of its potential toxicity to both humans and the environment. Human exposure can result in respiratory tract irritation, skin dermatitis, and allergic reactions, with long-term exposure potentially increasing the risk of cancer due to its genotoxic effects. Consequently, stringent controls on the production, use, and disposal of methyl orange are essential to mitigate its adverse environmental and health impacts, necessitating improved industrial practices, effective wastewater treatment technologies, and ongoing research into safer alternatives and mitigation strategies.

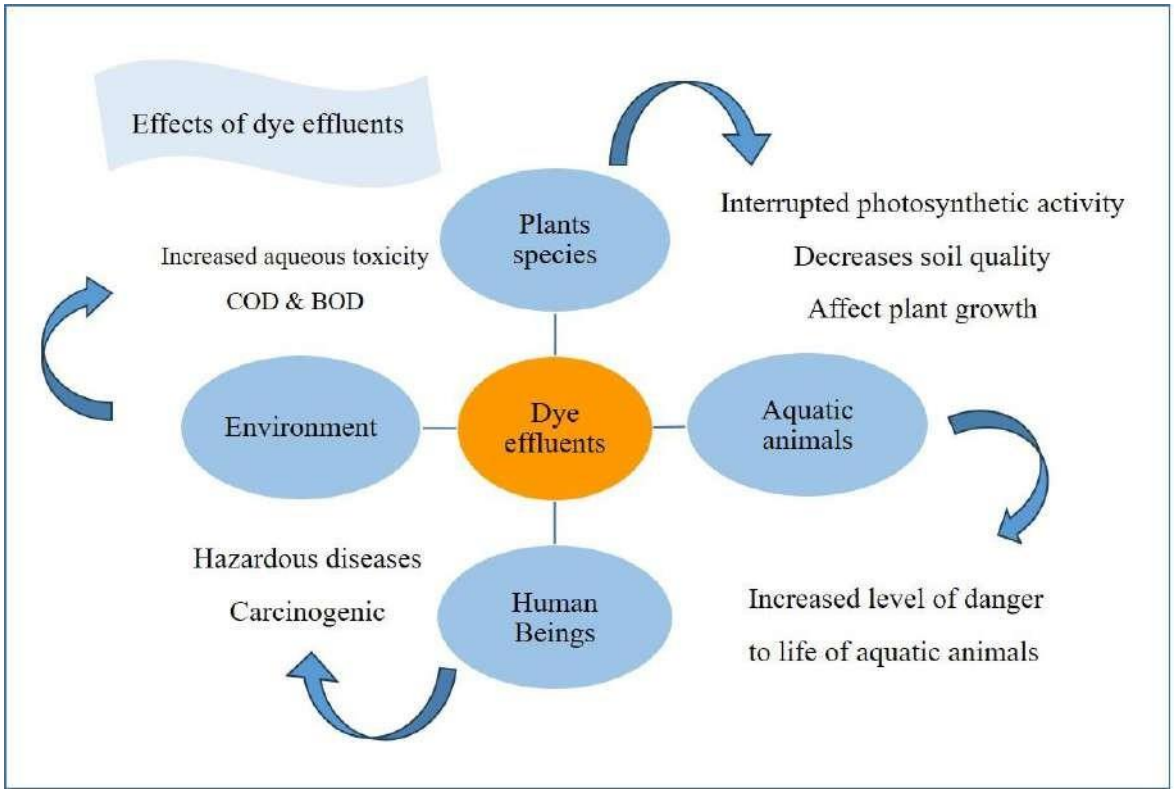


Figure 2.2: Implications of dye effluents on environment and the biota within.

2.4.1 Health Hazards

The potential health impacts of methyl orange on humans, while less studied, are noteworthy. Cases of human ingestion of methyl orange can result in gastrointestinal symptoms such as nausea, vomiting, and abdominal pain. El-Masry et al. (2020) reported acute poisoning symptoms in a 5-year-old child after accidental ingestion, with the child recovering after medical treatment. While some studies suggest that methyl orange is not a skin irritant or sensitizer, there are reports of contact dermatitis or allergic reactions in certain individuals. For example, a laboratory worker developed a rash after handling the dye (Tandon et al., 2018). Additionally, limited information is available on the health impacts of inhaling methyl orange. However, animal studies indicate that inhalation of high concentrations may cause respiratory irritation and damage. Meneses et al. (2013) found that rats exposed to high concentrations of methyl orange experienced respiratory irritation, lung damage, and inflammatory responses. Inhalation of the dye has been shown to cause respiratory irritation and damage to the respiratory tract, as demonstrated in experiments with rats (Xie et al., 2011). The dye's toxicity also extends to aquatic organisms and human blood lymphocytes, with potential genotoxic effects that may increases the hazard of cancer (Kocaman et. al., 2018). Overall, the available research suggests that ingestion of methyl orange can cause acute poisoning, while skin contact can lead to contact dermatitis or allergic reactions in some individuals. There is limited data on the health impacts of inhalation, highlighting the need for further research to fully understand the potential health risks of methyl orange exposure.

2.4.2 Effect on Aquatic Biota

The usage and disposal of methyl orange can severely impact aquatic environments. Numerous studies have investigated its effects on aquatic organisms and ecosystems. A study conducted by Chang and Wang (2012) found that methyl orange concentrations in a Taiwanese river exceeded acceptable limits, significantly affecting dissolved oxygen levels. Cheng and Chen (2011) demonstrated the toxicity of methyl orange to *Daphnia magna*, withEC50 value of 1.9 mg/L. Additionally, Dhara et al (2013) reported toxic effects on the fish species *Catla catla*, with an LC50 value of 0.015 mg/L. Chen et al. (2014) investigated the bioaccumulation of MO in fish, finding higher

concentrations in the liver and muscle tissue, which increased with exposure time and concentration. Guo et al. (2017) observed genotoxic effects in zebrafish, evidenced by DNA damage and nuclear abnormalities. These studies underscore the need for careful handling and disposal of methyl orange to prevent environmental contamination.

2.4.3 Terrestrial Impact

Although the impact of methyl orange on terrestrial environments is less understood, several studies highlight its potential toxicity to terrestrial biota. For instance, research by El-Ghany et al. (2020) demonstrated that high concentrations of methyl orange (100 and 200 mg/L) significantly reduced the growth and altered the biochemical parameters of the medicinal plant *Trigonella foenum-graecum*. Similarly, research conducted by Madkour et. al. (2019) found that exposure to high concentrations of methyl orange (50 and 100 mg/L) significantly reduced the growth and metabolic activities of the soil bacterium *Azotobacter chroococcum*. Furthermore, Yousaf et, al. (2021) reported that high concentrations of methyl orange (1000 and 2000 mg/kg) significantly reduced the survival and reproduction of earthworms (*Eisenia fetida*), indicating its potential hazard to soil fauna. These studies suggest that methyl orange can cause toxicity and adverse effects on terrestrial plants, microorganisms, and animals, necessitating further research to understand its environmental impact and potential for bioremediation.

2.5 Wastewater Treatment Strategies

Various physical and chemical treatment methods for decolorizing and detoxifying Methyl Orange (MO) dye are commercially available, including adsorption, filtration, precipitation, coagulation, chemical oxidation, photo-degradation, and electrolysis (Figure 2.3). However, these techniques have several disadvantages, such as the need for reagents, insufficient decolorization, high costs, higher energy consumption, and the cohort of sludge. Additionally, they may produce harmful by-products. Advanced oxidation processes (AOPs) utilize catalysts and/or oxidizing chemicals to detoxify and decolorize MO effectively, yet they are also associated with high energy and operational costs (Table 2.1). Azo dye metabolites, including MO, are known for their extreme resistance to degradation and have been linked to mutagenic, carcinogenic, and

teratogenic effects (Prabhakar et al., 2019). Therefore, it is imperative that wastewater containing MO be detoxified and decolorized before being discharged into the environment. Economical, environmentally friendly techniques are required for removal or degradation of dyes from wastewater.

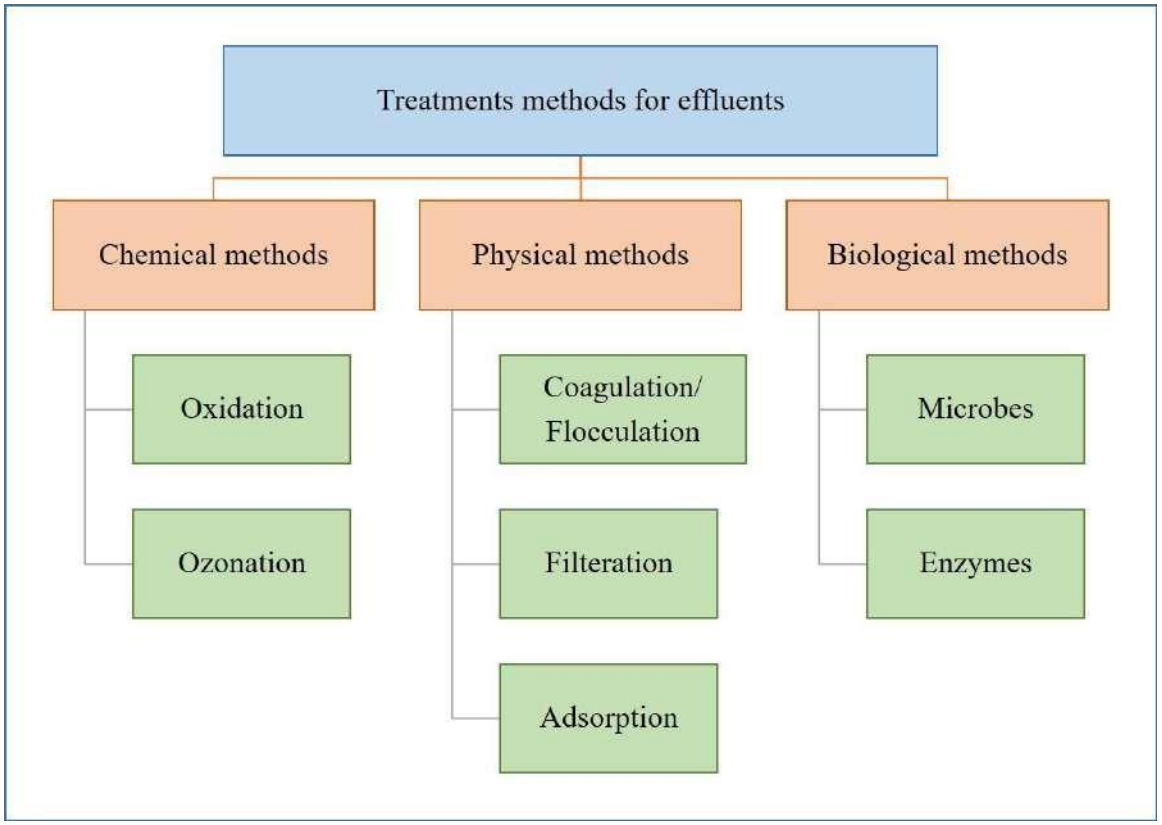


Figure 2.3: Depicting various methods implemented for the effective treatment of dye-dye-containing wastewater.

Table 2.1: Different Physico-chemical methods involved in dye removal

S. No	Physico-chemical methods	Applications	Advantages	Drawbacks	References
1	Ozonation	strong oxidant and disinfectant to eliminate color and odor, get rid of any traces of hazardous synthetic organic chemicals, and contribute to coagulation	Only applied in the gaseous phase	Very short half-life (20 min)	Al-Kadsi et al., 2004

2	Irradiations	It produces radical species, which react quickly with textile dyes and cause the chemical structure to be destroyed.	Lab-scale oxidation that works well	Needs a large amount of dissolved oxygen	Chen et al., 2006
3	Ion Exchange	Charged ions produced as a result of dye degradation are collected by an ion-exchange resin.	No adsorbent loss during regeneration	Not suitable for all dye types	Raghu and Basha, 2007
4	Silica gel	Much dye degradation is facilitated by the high surface area, strong SiO2 particle adsorption, and superior sedimentation ability.	efficient for removing basic dyes	Side reactions prevent	Zhao et al., 2008
5	Magnetic Nanoparticles	Waste-water Treatment	Provide a surface to which specific ligands for a contaminant can be attached, ensuring stability and protection from oxidation.	In vitro Cytotoxicity	Costa et al., 2011
6	Fenton's reagent	oxidation in situ with Fenton's chemical formula. At the amounts generated, the reaction's byproducts are not harmful	Suitable for soluble and insoluble dyes	Sludge production	Daniela and Carmen, 2012
7	Electrokinetic	It causes various chemical reactions by passing an electric current	Economically feasible	High sludge formation	Pereira et al., 2012

	coagulation	current across the electrodes. The cutting-edge cathodic electron transfer takes the place of the reducing agent.			
8	Carbon Nanotubes	"Point-of-use highly biodegradable pollutants, such as antibiotics and pharmaceuticals."	Highly reusable, bactericidal, and accessible sorption sites	Excessive production costs and the associated health risks.	Qu et al., 2013
9	Nano Adsorbents	Point-of-use, removal of bacteria, heavy metals, and organics,	high adsorption rates, higher specific surface, small footprint	Higher production costs	Gehrke et al., 2015
11	Membranes and membrane processes	Wastewater and water treatment techniques in every field	reliable, mostly automated procedure	Compared to higher energy demand, Concentrated Sludge production	Gehrke et al., 2015
12	Nanoparticles (Ag, TiO_2, ZnO, layered ceramic filters)	Small diffusion resistance, higher adsorption capacity, larger surface area, faster adsorption equilibrium	Offers an effective and economical way to environmental bioremediation protection	Leaching problem	Hosseinnia et al., 2010
14	Peat	Peat's unique quality is its capacity for cationic exchange.	Good adsorbent due to its cellular structure	Compared to activated carbon, the specific surface area for adsorption	Ong et al., 2011

				are less	
15	Photochemical with H_2O_2	The production of hydroxyl radicals, which are extremely reactive and non-selective, can break down a variety of organic contaminants	Absence of sludge production	Formation of byproducts	Lodha et al., 2010

Biological methods have emerged as promising alternatives for the treatment of dye-contaminated wastes-waters. These methods involve the bioconversion of organic pollutants into stable, non-toxic end products (Youssef et. al, 2016; Huo et. al., 2013). Biological treatment methods, including microbial degradation, enzymatic degradation, and phytoremediation, are environmentally friendly, less energy-intensive, and effective in removing dyes like MO. Microbial degradation involves different microbial species and metabolic pathways to break down the dye molecules, leading to complete decolorization and detoxification without generating harmful by-products. These biological methods are not only cost-effective but also sustainable, making them ideal for large-scale applications in treating MO-contaminated wastewater.

2.5.1 Microbial Remediation of Methyl Orange

Traditional physicochemical methods coagulation, flocculation, and membrane filtration, while commonly employed, often encounter significant limitations. These include reagent requirements, substantial energy, high operational costs, incomplete decolorization, and the production of secondary pollution in the form of sludge and harmful by-products. Advanced Oxidation Processes (AOPs), including techniques like ozonation and photo-catalysis, provide more effective alternatives but are often constrained by their expense and high energy consumption.

Bioremediation technologies, comprising microbes, enzymes, and plants, present a more sustainable approach, offering several advantages over physicochemical and methods. These biological methods are generally cheaper, environmentally friendly,

generate less sludge, require less water, and produce non-toxic metabolites. Among microorganisms, bacteria exhibit a faster rate of MO decolorization compared to fungi. Various bacterial strains have been identified for their ability to efficiently remove MO from wastewater. Notably, bacterial consortia, which consist of multiple strains, have demonstrated greater efficacy than single strains due to their ability to attack dye molecules at multiple sites or to utilize the metabolites produced by co-existing strains for further decomposition. For example, bacterial consortia have been shown to decolorize MO more effectively than isolated strains (Gomare et al., 2018). Bacterial biofilms represent a promising enhancement in the detoxification process. These biofilms consist of surface-associated bacterial cells that produce extracellular polymeric substances (EPS), providing protection from environmental stressors such as high concentrations of chemicals, temperature fluctuations, pH variations, and salinity. Biofilm cells also produce signal molecules and enzymes more efficiently than free-living bacteria and facilitate enhanced genetic material exchange. Despite their advantages, potential of biofilm in improving detoxifications only been recognized and applied, warranting further investigation.

In addition to microbial approaches, phytoremediation has emerged as a promising method for MO removal from contaminated environments. Plants such as *Typha latifolia, Lemna minor*, and *Pistia stratiotes* have been shown to effectively remove MO through processes of adsorption and phyto-degradation. Recent advancements in genetic engineering have led to the development of plants capable of expressing MO-MO-degrading enzymes, offering a novel solution for MO remediation. However, the application of genetically modified plants in field conditions requires further research to optimize degradation conditions and ensure ecological safety. Moreover, techniques such as bio-stimulation and bio-augmentation have been employed to enhance microbial degradation of MO. Bio-stimulation involves the addition of nutrients to the contaminated environment to promote the growth of indigenous microbial populations capable of degrading MO. In contrast, bio-augmentation involves the introduction of an exogenous microbial strain in the contaminated environment to enhance degradation. Studies have demonstrated that the combination of bio-stimulation and

bio-augmentation can significantly enhance MO degradation in contaminated environments (Makhdoumi-Kakhki et al., 2017).

Overall, while methyl orange remains a valuable tool in various industrial and scientific applications, its potential environmental, health hazards necessitate the development and implementation of effective wastewater treatment strategies. Bioremediation, leveraging naturally occurring microorganisms and plants, offers a sustainable and cost-effective solution to mitigate the pollution caused by MO and similar dyes, thereby protecting both environmental and human health. Further research is essential to optimize these bioremediation techniques and develop comprehensive, sustainable strategies for the treatment of MO-contaminated environments.

2.5.2 Decolorizations and Detoxifications of MO

Decolorization and detoxification of (MO) are critical steps in treating MO- MO-contaminated environments due to the dye's potential carcinogenic, teratogenic, and mutagenic effects, and its resistance to degradation (Chen et. al, 2011; Prabhakar et al., 2019). Various physical, chemical, and biological methods have been developed to address this issue. Physical methods, such as adsorption, coagulation, and filtration, are effective for removing MO from contaminated water but do not achieve complete removal or degradation of the dye. Chemical methods, including oxidation and reduction, can degrade MO into non-toxic products but may generate hazardous by-products that pose additional environmental and health risks. (AOPs), While effective, they are expensive and energy-intensive.

Biological methods, particularly microbial degradation, enzymatic degradation, and phytoremediation, offer alternatives due to their cost-effectiveness and environmental friendliness. Microbial degradation of MO involves complex processes utilizing various microbial species and metabolic pathways. Notably, bacteria are generally faster than fungi in decolorizing MO, and bacterial consortia have proven more effective than single strains by attacking the dye molecules at multiple positions or utilizing metabolites formed by co-existing strains for further decomposition (Gomare et al., 2018).

Parshetti et al. (2009) reported 100% decolorization of MO (50 mg/L/) under static conditions, with optimal decolorization at pH 6.8 and 30°C. The growth medium containing yeast extract was most effective in promoting the growth of *Kocuria rosea* (MTCC 1532), achieving the highest decolorization. Metabolites identified using FTIR and GC-MS, including 4-amino sulfonic acid and N, N-dimethyl p-phenylenediamine, were non-toxic to both bacteria (*Pseudomonas aeruginosa*, *K. rosea*, and *Azotobacter vinelandii*) and plants (*Phaseolus mungo and Triticum aestivum*) and (Parshetti et al., 2009). Another study by Kishor et al. (2021) identified *Pseudomonas aeruginosa* RKS6 from textile industries' waste-water and sludge samples, which achieved over 99% decolorization and 96% reduction of Total Organic Carbon (TOC) of MO (100 mg/l) within 12 hours under static conditions at 30°C and pH 7. The bacterium produced manganese peroxidase (MnP) enzyme, as confirmed by SDS-PAGE analysis, and LC-MS analysis revealed non-toxic degradation products, promoting 90% seed germination in treated wastewater (Kishor et al., 2021). Recent research by Haque et al. (2021) demonstrated the effectiveness of bacterial biofilm consortia in decolorizing and detoxifying MO. Four consortia showed significant decolorization, with the consortium C3 (comprising *Escherichia coli* ENSD101, *Enterobacter ludwigii* ENSH201, and *Bacillus thuringiensis* ENSW401) being the most effective. Optimal conditions were pH 7.0 and 28°C, with certain metal ions enhancing decolorization. The azo bond was completely eliminated within 60 hours, and the resulting metabolites were non-toxic (Haque et al., 2021).

Recently the capability of *Stenotrophomonas acidaminiphila* EFS1 to remove and detoxify MO, achieving 80% decolorization within 24 hours under optimal conditions was investigated. FTIR analysis indicated that decolorization resulted from the breakdown of azo bonds, forming non-toxic metabolites. These findings underscore the potential of microbial and phytoremediation approaches for the sustainable treatment of MO-contaminated wastewater (Yang et al., 2022). The effectiveness of decolorization and detoxification of MO is inclined by factors temperature, pH, nutrient availability, and the presence of pollutants. Optimal conditions for microbial degradation of MO generally include a pH range of 6.0 to 8.0 and a temperature range of 20°C to 35°C. Nutrient addition, particularly carbon and nitrogen sources, can

enhance microbial activity. However, the presence of other pollutants, organic compounds, and heavy metals can inhibit the process. Overall, the development of sustainable and cost-effective bioremediation strategies is essential for the efficient treatment of MO-contaminated environments.

Table 2.2: Previous studies focusing on microbial remediation of Methyl orange

S. No.	Micro-organism used	Dye Concentration	pH/ Temperature	Bioremediation focus	References
1.	*Pseudomonas spp.*	500 mg^{-1}	6–10/ 30–40°C	Decolorization (10–94%)	Shah et al., 2013
2.	*P. aeruginosa*	20 ppm	9/ 40 °C	Biodegradation/ Detoxification (88.37%)	Ikram et al., 2022
3.	*Pseudomonas aeruginosa*	20 ppm	7/ 37 °C	Bio-Mineralization/ Decolorization (88.23%)	Khan et al., 2022
4.	*Bacillus stratosphericus* SCA100 7	150 mg/L	7/ 35°C	Decolorization/ Degradation (100%)	Akansha et al., 2019
5.	*Micrococcus yunnanensis*	100 mg L^{-1}	7/ 30°C	Decolorization (90%)	Carolin et al., 2021
6.	*Stenotrophomonas acidaminiphila* EFS1	20 mg/L	7-7.2/ 37°C	Decolorization (80%)	Yang et al., 2022
7.	*K. rosea* (MTCC 1532)	50 mg L^{-1}	6.8/ 30°C	Decolorization 100%	Parshetti et al., 2009
8.	*Pseudomonas aeruginosa*	100 mg/L	7/ 30°C	Detoxification/ Decolorization >99%	Kishor et al., 2021

Table 2.3: Literature Review table of different dye-degrading organisms

Sr. No	Name of Organisms	pH	Temp.	Kinetic Techniques	Time of decolorization	Remove	Method	References
1	*Bacillus stratospheric* SCA1007	7.0	35ºC	UV-vis and FTIR	12hrs	150mg/ L	Degrade	Akanksh a et al.,2019

2	*Aeromonas hydrophila*	7.0	35ºC	FT-IR and GC–MS	24hrs.	20ppm	Degradation	Velusamy et al., 2021
3	Hog Plum Peel and mixed Bacterial strains	8.0	37ºC	Langmuir Isotherm	60min.	500mg/ ml	Adsorption	Rumky J. et al.,2013
4	*Aeromonas sp*. Strain DH-6	3.0-7.0	5-45ºC	GC-MS, HP-LC	12hrs.	100mg/ L	Degradation	Lin-Na Du et al., 2015
5	*Aeromonas veronii GRI*	7	37ºC, 150rpm	-	24hrs.	1000mg/L	Degradation	Mniff et al., 2015
6	*Micrococcus yunnanensis*	7	37ºC	FT-IR and GC-MS	24hrs.	100mg/ L	Degradation	Carolin et al., 2020
7	*Pseudomonas* spp. ETL-1982	4-10	30-40ºC	UV-vis	24hrs.	40-120mg/ L	Biosorption	Shah et al., 2013
8	*Kocuria rosea* (MTCC 1532)	6.8	30ºC	FTIR and MS	24hrs.	50mg/ L	Degradation	Parshetti et G.K et. al., 2009
9	Polyaniline e-coated bacterial cellulose (PANi@BC)	7	37ºC	Pseudo-second order Kinetics and Langmuir Isotherm model	10hrs.	1000mg/L	Adsorption	Jahan et al., 2020

10	*Pseudomonas fluorescens* and *Acinetobacter baumannii* mixed culture	7	30ºC	-	57hrs.	150mg/ L	Decolorization	Ghanem et. al., 2011.
11	*Shewanella oneidensis* MR-1	6.5		Electron transfer, Metal respiration pathway 13Aerobic respiration	7hrs.	200mg/ L	Anaerobic bio Decolorization Degradation	Cai. P et al., 2011
12	*Rhodococcus* strains UCC 004	-	-	UV–vis spectrum	48hrs.	0.5g/L	Degradation	Maniyam et al., 2018
13	*Klebsiella* spp.	4 to 9	30 to 42 °C	UV–vis spectrum	48hrs.	100mg/ L	Degradation	Cui et al., /2014
14	*Oedogonium subplagiostomum , (Algae)*	6.5	30ºC	Adsorption isotherm , kinetics and thermodynamics	132hrs.	500mg/ L	Degradation/ Biosorption Decolorization	Marutha nayagam . A et al., 2020

15	Periphyton (epiphyton, epilithon, or metazoan Phyto) includes Algae, cyanobacteria, and some unicellular diatomic species.	-	-	pseudo-second order kinetics, FTIR/ GC-MS	72hrs.	500mg / L	Biosorption	Shabbir et al., 2016
16	*Pseudomonas aeruginos MZ*52073 0	7	30ºC, static	FTIR, LC-MS	12hrs.	100mg/ L	Degradation	Kishore et al., 2021
17	*Sphingomonas paucimobilis, Bacillus cereus ATCC145 79, Bacillus cereus* ATCC117 78 *(*Consortium)	7	30ºC	UV-vis, FTIR, NMR	48hrs.	750ppm	Degradation	Ayed et al., 2010
18	*Nesterenkonia lacusekhoensis EMLA3*	11.5	37ºC, Agitation speed 200 rpm	-	192hrs.	100mg/ L	Degradation	Prabhakar. Y et al., 2020
19	*Franconibacter sp.* 1MS	7.0	37ºC	UV-vis, FT-IR and GC-MS	120hrs.	100mg/ L	Degradation	Baena-Baldiris. D et al., 2020

20	*Bacillus sp.*PS	Neutral	32ºC, 140rpm	-	72hrs.	3ml from 1000mg /L	Degradation	Pourbabae et al., 2006
21	*Bacterial consortium* (*Sphingomonas paucimobilis, Bacillus cereus* ATCC145 79, *Bacillus cereus* ATCC117 78)	7	30ºC	FTIR, NMR, spectroscopy	48hrs.	750ppm	Degradation	Ayed L. et al., 2010
22	Mixed bacterial culture *(Bacillus* sp. strain AK1, *Lusinibacillus* sp. Strain AK2 *and Kertersia* sp. Stain VKY1	7	35ºC	HPLC and FTIR	24hrs.	800mg/ L	Degradation	S. Masarbo Ramesh et al., 2008

CHAPTER 3

HYPOTHESIS

CHAPTER 3 – HYPOTHESIS

In the era of escalating environmental challenges posed by industrial pollution, the need for innovative and sustainable resolutions is mandatory. The current research study hypothesizes that isolating and identifying specific microbial strains from textile effluent samples collected in Panipat's industrial sector will reveal microbes capable of efficiently degrading Methyl Orange azo dye. This hypothesis is based on the pervasive use of Methyl Orange in the textile industry, which has been extensively documented through surveys and site inspections of Panipat's industrial areas. The frequent discharge of this sulfonated azo dye into natural water bodies raises significant ecological and eco-toxicological concerns. It is anticipated that microbial strains adapted to these polluted environments possess specialized enzymatic mechanisms for breaking down complex azo-dye structures, thereby offering a natural and effective solution for bioremediation. The study aims to isolate these microbial strains and comprehensively characterize their biological and physico-chemical properties. By proceeding with this, it seeks to determine their efficiency in degrading Methyl Orange under various conditions. Once identified, these strains will be employed in the development of bio-filters designed for in-situ bioremediation. The expectation is that these bio-filters, utilizing the potent dye-degrading capabilities of the isolated microbes, will significantly improve the treatment of textile effluent. This improvement will be measured by a reduction in pollutant levels and an overall enhancement in water quality.

In addressing the broader ecological implications, the study also aims to answer key research questions related to the impact of industrial effluent on natural water bodies. This includes assessing how untreated or inadequately treated effluents affect the ecosystem and contribute to eco-toxicological issues. By characterizing the biological and physico-chemical properties of the effluent, the study will provide insights into the effectiveness of existing treatment processes and highlight the need for stricter environmental regulations.

Additionally, the study will employ various metrics to assess the water quality of textile effluent and the receiving water bodies, specifically focusing on an ancient rivulet drain that runs through Panipat city. This drain serves as a critical point of reference for understanding the extent of pollution or its impact on local water bodies. By comparing the water quality before and after the implementation of microbial bio-filters, the study will quantify the improvements brought about by bioremediation efforts. Ultimately, the successful identification and application of these microbial strains in bio-filters is expected to offer a sustainable and practical solution for treating industrial wastewater. This would not only help in achieving compliance with environmental regulations but also contribute to the restoration and preservation of aquatic ecosystems. Improved water quality would have far-reaching benefits for both public health and the environment, demonstrating the potential of bioremediation as a viable approach to managing industrial pollution. This study, therefore, represents a critical step towards developing innovative and eco-friendly solutions for one of the most pressing environmental challenges faced by the textile industry today.

CHAPTER 4

OBJECTIVES

CHAPTER 4 – OBJECTIVES

1. Isolation of a new bacterial strain capable of removing methyl orange from aqueous solution and morphological, biochemical, and molecular characterization of the isolated strain.
2. Statistical optimization of the Physico-chemical parameters for rapid removal of methyl orange from aqueous solution.
3. Assessment of the possible mechanism involved in the dye removal process.
4. Toxicity analysis of the treated solution on selected agriculturally important crop seed germination.
5. Immobilization of the isolated strain for possible remediation of textile industry effluent.

CHAPTER 5

MATERIALS AND METHODS

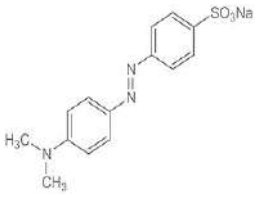
Chapter 5 - Materials and Methods

Objective 1: Isolation of a new bacterial strain capable of removing methyl orange from aqueous solution and morphological identification, biochemical identification, and molecular characterization of the isolated strain.

5.1 Chemicals and Dye

The analytical-grade chemicals used in this study were as specified. The supplier of methyl orange was Sigma Aldrich in Bangalore, India. Methyl Orange is a pH indicator with the IUPAC name Sodium 4-[(4-dimethylamino) phenylazo] benzenesulfonate. Its maximum absorption wavelength (λmax) is 465 nm, and its molecular weight is 327.33 g/mol. Its chemical formula is $C_{14}H_{14}N_3NaO_3S$. The structure of Methyl Orange includes an azo group (-N=N-) connecting two aromatic rings, with one ring bearing a dimethyl amino group and the other a sulfonate group (Carolin et al., 2021) (Table 5.1). Nutrient media, including Nutrient Agar and Nutrient Broth, as well as nitrogen and carbon sources, were obtained from HI Media, Mumbai, India.

Table 5.1: Properties of methyl orange dye utilized for experimental purposes.

Name	**IUPAC Name**	**Molecular weight**	**Λmax**	**Chemical formula**	**Chemical Structure**
Methyl Orange (MO)	sodium 4-[(4dimethylamino) phenylazo] benzene sulfonate	327.33g/mol	465nm	$C_{14}H_{14}N_3$ Na O_3 S	

5.2 Sample Collection

Effluent samples of wet sludge, dry sludge, and soil were collected from an industrial area in Panipat, Haryana (Figure 5.1). Each sample was collected and kept at room temperature in sterile glass containers. samples were tested within 24 hours of collection to ensure the validity and reliability of the findings.

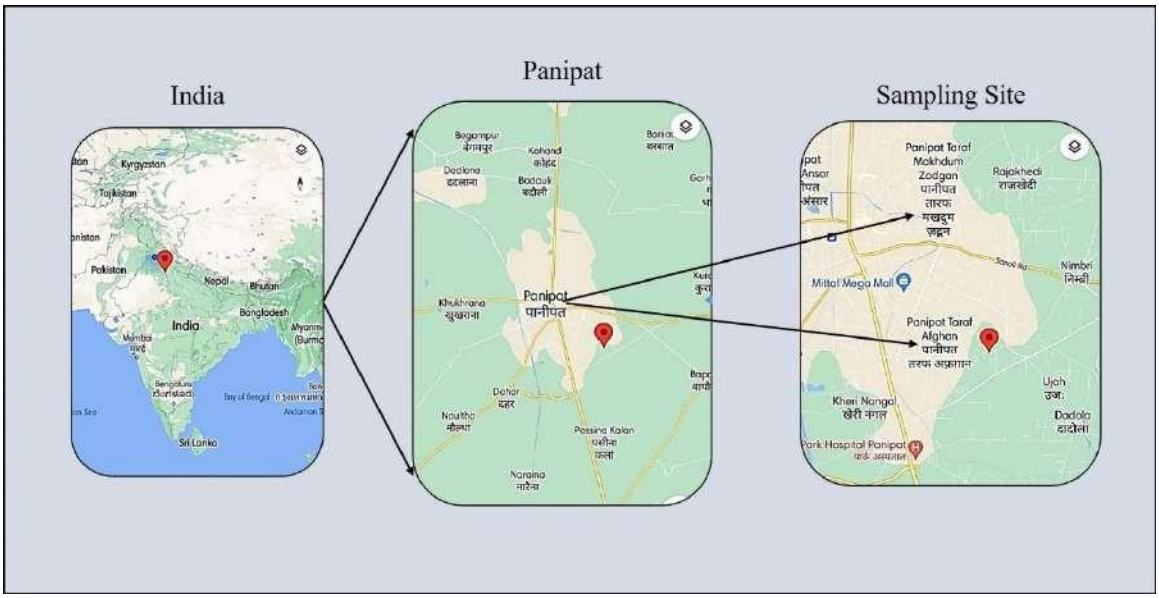


Figure 5.1: Depicting the location of the sampling site

5.3 Isolation of Strain and Culture Conditions

Textile wastewater was taken from the industrial area of Panipat, Haryana, India, to isolate bacteria capable of removing dyes. A 250 ml flask with 100 ml of nutrient broth was inoculated with a 10 ml sample of textile effluent and cultured at 35°C for 48 hours. Following incubation, 1 ml of the sample was taken and subjected to Serial dilution up to 10^{-5}. The diluted samples were then poured on Petri dishes containing NA amended with 100 mg/L of methyl orange using the spread plate methods.

After microbial growth on the plates, eleven strains designated J1 to J11 were isolated based on their morphological characteristics and sub-cultured onto nutrient agar plates. Screening of these strains identified J4 as the most effective in removing methyl orange

dye. The bacterial strain J4 was maintained on nutrient agar slants, and inoculum preparation followed the method described by Goswami et al. (2012).

5.3.1 Removal of Methyl Orange Using Strain Bacillus cereus J4: Batch Decolorization Experiments

Samples were collected from dyeing zones in Panipat, Haryana, India, and then spread over an NA plate after being serially diluted (Velusamy et al., 2022). Biochemical identification, morphological identification, and molecular characterizations, including 16S rRNA gene sequencing performed by IMTECH Chandigarh, India, confirmed the strain as *Bacillus cereus* J4. The bacterial strain J4's 16S rRNA sequences have been added to GenBank with **accession number OQ392442**.

De-colorization of the Sulfonated Azo dye MO was demonstrated in 100 ml bottles containing 50 ml NB inoculated with *Bacillus cereus* J4. The inoculum size was adjusted to an OD of 1.0 (λ max = 600 nm, 1.50×10^6 cells/mL) and incubated at agitation speed of 150 rpm and 35°C. Culture conditions were subsequently used in sequential experiment. After the bacterial biomass was separated by centrifugation, two-milliliter aliquots were taken out, and the OD of the supernatant was noted. Figure 5.2 shows the calibration of an MO concentration vs absorbance curve to estimate the λ max of the test dye and record a reduction in MO dye concentration (Carolin et al., 2021; Velusamy et al., 2022).

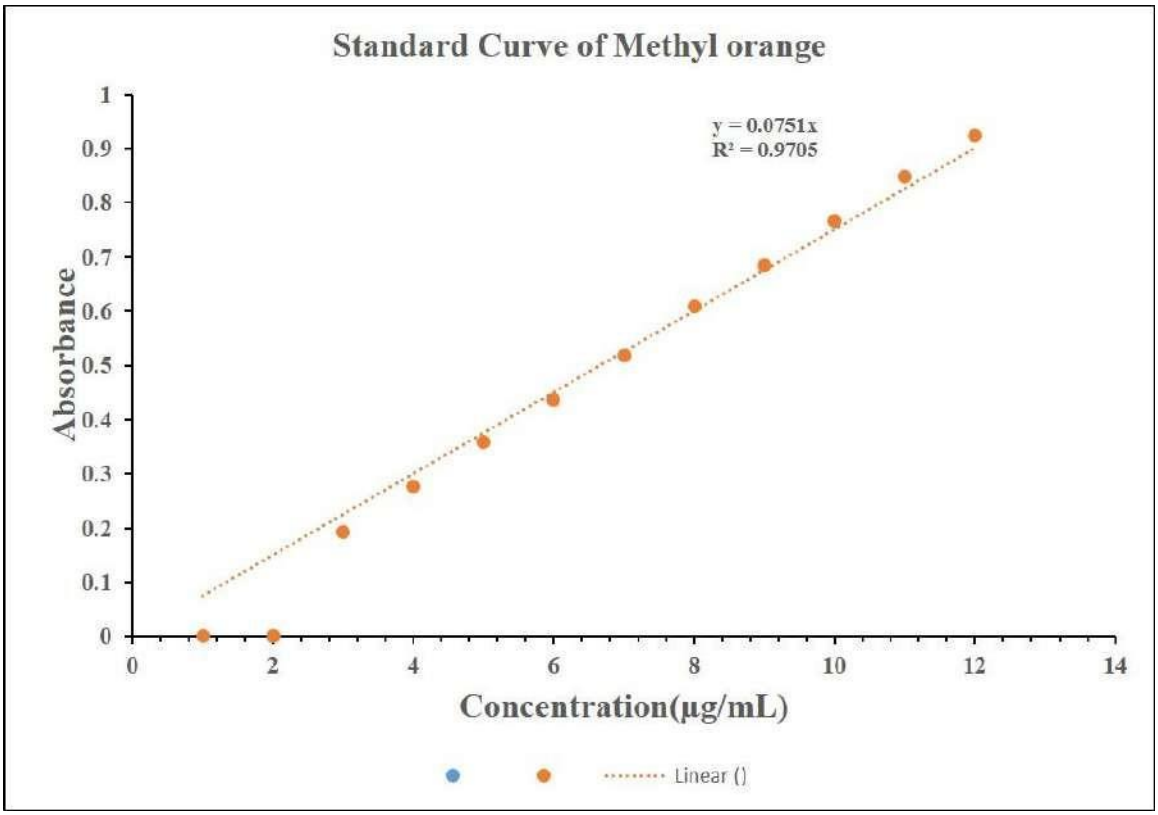


Figure 5.2: Standard curve of methyl orange.

The de-colorization activity of the MO was assessed by calculating the absorbance of the solution two times (before and after the treatment). The absorbance (optical density, OD) was measured using a spectrophotometer. The percentage de-colorization and the concentration of dye removed were calculated by using the formula given below (Dos et. al., 2007; Velusamy et. al., 2022):

Dye de-colorization (%) = Initial OD - Final OD / Initial OD×100

Where Initial OD is the optical density of the dye solution at 0 time (before treatment). The final OD is the optical density after treatment.

These calculations were performed to quantify the effectiveness of the de-colorization process, providing a clear measure of the dye removal efficiency achieved by the bacterial strain *Bacillus cereus* J4.

Objective 2: Statistical optimization of the Physico-chemical parameter

5.4 Optimization of Physicals and Chemical Parameters for Removal of MO

Optimization of the operating conditions is crucial to achieving maximum de-colorization efficiency for azo dyes like Methyl Orange (MO) (Chowdhury et al., 2022). The effectiveness of biological treatment systems is significantly influenced by various physicochemical parameters (Dutta et al., 2022). This study optimized key physical parameters, including initial dye concentrations, inoculum percentage, incubation time, temperature, pH, and shaking rate (Kishore et al., 2021), as these factors profoundly affect microbial degradation efficiency.

5.4.1 Initial Dye Concentration

Experiments were conducted to optimize the initial dye concentration for maximum decolorization efficiency of MO. The study involved varying the initial concentrations of the dye in a range from 10 ppm to 100 ppm. Dyes with concentrations ranging from 10 ppm to 100 ppm were added to various flasks that contained nutritional medium. Pure bacterial cultures were then inoculated into these flasks and incubated at a temperature of 37ºC with the pH maintained at 7. During the incubation period, the samples were periodically analyzed to measure both the growth of the bacterial cultures and the percentage of dye decolorization (Ecer et .al, 2021). This approach allowed for the determination of the most effective dye concentration for optimal microbial degradation and dye removal from the solution.

5.4.2 Inoculum Percentage

To optimize the inoculum percentage for the de-colorization of MO, a series of experiments were conducted using flasks filled with 50 mL of nutrient broth and 50 mg/L of dye. The pH of the medium was carefully adjusted to 7 to ensure an optimal environment for bacterial activity. Different amounts of bacterial inoculum were introduced into the flasks at varying percentages: 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%. These flasks were then incubated at an optimal temperature determined from prior experiments, ensuring that conditions favored maximum microbial activity. During the incubation period, samples

were taken at regular intervals, centrifuged to remove the bacterial biomass, and the supernatant was analyzed to determine the percentage of dye de-colorization (Fu et al., 2023). This systematic approach allows for the identification of the most effective inoculum percentage that maximizes the microbial degradation of the dye, providing critical insights into the scaling and efficiency of the bioremediation process.

5.4.3 Incubation Time

To determine the optimal incubation time for the removal of MO dye, a series of experiments were performed. A flask (250 ml) containing 50 ml of NB, along with 50 ppm of MO dye solution, was prepared and inoculated with bacterial cultures that had been grown for 24 hours to ensure their active metabolic state. The flask was then incubated at a constant temperature of 37ºC to facilitate microbial activity. Samples were collected at various time intervals (8h, 16h, 32h, 40h, 48h, 56h, and 72h). To isolate the bacterial cells from the supernatant, samples of the growth media were carefully extracted and then centrifuged at regular intervals. The clear supernatant was then analyzed using spectrophotometry to measure the degree of dye decolorization, which indicates the efficiency of the microbial degradation process over time (Mahar et al., 2021). This methodical approach allows for the identification of the most effective incubation period, ensuring maximum dye removal.

5.4.4 Temperature

To optimize the temperature conditions for the de-colorization of MO dye, experiments were conducted across a range of temperatures (Nong et al., 2023). Flasks with a capacity of 250 ml contain 50 ml of nutrient broth, and are mixed with 50 ppm of MO dye solution. These flasks were then inoculated with bacterial cultures that had been cultivated for 24 hours to ensure they were in an active growth phase.

The inoculated flasks were incubated at various temperatures, specifically set at 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C, to evaluate the impact of temperature on the dye degradation process. Samples were systematically collected at predetermined times

intervals from each flask to monitor the progress of the de-colorization process. Each collected sample was centrifuged to separate the bacterial cells from the supernatant. The supernatant was then analyzed using spectrophotometry to determine the extent of dye de-colorization at each temperature (Ecer et al., 2021). This analysis provides a percentage de-colorization value, which is crucial for understanding how different temperatures influence microbial activity and efficiency in breaking down the dye.

5.4.5 Initial pH

To determine optimal pH for MO dye de-colorization, experiments were performed in 250 ml flasks, each containing 50 ml of nutrient broth and a 50-ppm dye solution. The pH levels were adjusted from 4 to 9. These solutions were inoculated with 24-hour-old bacterial cultures and incubated at an optimal temperature. Samples were taken at regular intervals, centrifuged to separate bacterial cells, and the supernatant was examined using a digital spectrophotometer to measure de-colorization (Ecer et al., 2021; Chowdhury et al., 2022). The percentage of de-colorization at each pH level was calculated to identify the most effective pH for dye removal. This experiment helps optimize pH conditions for efficient bioremediation.

5.4.6 Agitation Speed

The impact of agitation speed on the bacterial de-colorization of MO dye was examined by conducting tests at various shaking speeds, ranging from 50 rpm to 200 rpm. These experiments utilized a screening medium with a 50-ppm concentration of MO dye. Optimal conditions for the tests were maintained, including an inoculum percentage of 1.5%, an optimized incubation time (48 hours), an initial pH of 7.0, and a temperature of 37°C. At specific intervals, supernatant samples were taken from the flasks, centrifuged to remove bacterial cells, and then analyzed spectrophotometrically to measure the extent of dye de-colorization (Haque et al., 2021). This study aims to determine the most effective agitation speed to enhance microbial activity and maximize the de-colorization efficiency of the dye.

5.5 Chemical Parameters

5.5.1 Extra Carbon Sources

The influence of additional carbon sources on the de-colorization efficiency of MO was investigated by supplementing the nutrient broth with five carbon sources (D-fructose, sucrose, maltose, D-glucose, and mannitol) each at a concentration of 50 ppm MO. The nutrient broth containing these carbon sources was inoculated with a fresh culture and incubated at 37°C. To evaluate the decolorization process, aliquots were withdrawn from the flasks at optimized intervals. These samples were then centrifuged to separate the bacterial cells from the supernatant (Fu et al., 2023). The supernatant was analyzed spectrophotometrically to calculate the percentage of de-colorization.

5.5.2 Extra Nitrogen Sources

To explore the impacts of various nitrogen sources on the de-colorization efficiency of MO, different organic and inorganic nitrogen compounds were incorporated into the media containing 50 mg/L MO. The nitrogen sources that were examined included peptone, sodium nitrates, ammonium nitrate, ammonium sulfate, and urea. After preparing the media with these nitrogen sources, the flasks were sterilized and inoculated with a 24-hour-old bacterial culture. The inoculated flasks were then incubated at 37°C to facilitate the microbial activity. Samples were collected at different time intervals to observe the dye removal process. These samples were centrifuged to separate the bacterial cells from the supernatant. The supernatant was analyzed spectrophotometrically to calculate the percentage of de-colorization (Fu et al., 2023).

5.6 Statistical Optimization using PB and RSM method

To optimize the removal percentage of Methyl Orange (MO) using *Bacillus cereus* J4, a combination of the PB method and RSM was employed (MA et al., 2022; Ullah et al., 2023). DOE (Version 7.5.1, Stat-Ease, Minneapolis, USA) facilitated this optimization process.

5.6.1 Plackett-Burman Method for Significant Factor Screening

The Plackett-Burman (PB) was initially used to identify the significant parameters that influence the removal of MO (Ullah et al., 2023). The PB design included three central points to assess the effects of various culture medium components and fermentation conditions. The independent physical variables studied were initial dye concentration (10-100 mg/l), Inoculum percentage (0.5-2.5% v/v), Incubation time (8-72 hours), pH (4-9), Temperature (25-50ºC), and Agitation speed (static to 200 rpm).

Chemical factors considered were extra nitrogen sources (ammonium chloride, ammonium sulfate, Peptone type I-bacteriological, urea, and L-tryptophan) and extra carbon sources (maltose, glucose, lactose, sucrose, dextrose, and fructose) at concentrations of 0.5 % to 3%. Preliminary results indicated that urea and glucose produced the best outcomes and were thus selected for further study. After conducting COVT (changing one variable at a time) trials, variables were set at fixed levels. Using the Central Composite Design (CCD), four important parameters from this experiment were found to need more modification (optimization).

5.6.2 Applications of RSMs to Optimize MO Removal by Bacillus cereus J4

The CCD was used to apply RSM to analyze the optimal conditions for dye decolorization. This method helps in quantifying the correlations between the input elements and the measured results (Ibrahim et al., 2022). The optimization process involved 3 main steps: conducting a statistically designed experiment, analyzing the coefficients in mathematical models to predict responses, and examining the model's adequacy.

The relationship can be expressed as:

$Y = f(X_1, X_2, . \quad X_n)$

Where 'Y' represents the response (removal% %), and $(X_1,..X_2,. . . .X_n)$ are independent variables.

5.6.3 Design of Experiments

The four significant factors identified were Initial dye concentration(A), Initial pH(C), Temperature(B), Extra nitrogen source(D)

To maximize the percentage of dye removal, the CCD used high, medium, and low levels, denoted by code values of +1, 0, and -1 for the variables. The ranges of input factors were determined using the COVT experimental findings. The total number of tests needed for the four independent variables is:

$N = 2n + 2n + nC = 24 + 2 \times 4 + 6 = 30$ (3)

The CCD comprises two (2n) factorial runs with two (2n) axial runs and nc central runs.

Objective 3: Assessment of the possible mechanisms involved in the dye removal process.

Field Emission Scanning Electron Microscopy (FESEM), (FTIR), and High-Performance Liquid Chromatography (HPLC) were utilized to analyze the components and characteristics of the samples (Hashemi et al., 2022).

5.7 Field Emission Scanning Electron Microscopy (FE-SEM)

FESEM is a powerful analytical technique used for high-resolution imaging of surface structures. This technique employs a focused beam of electrons emitted from a field emission source, which interacts with the sample surface to produce detailed images (Lewczuk and Szyryńska, 2021). FE-SEM allows for the examination of surface topography, composition, and microstructural details with exceptional clarity, making it ideal for studying nanomaterials, biological specimens, and other complex materials. The analysis was performed in the CIF, Lovely Professional University, Punjab, India. The equipment utilized was the JOEL FESEM, a versatile high-resolution instrument with an SEI resolution of 1.0nm at 15 kV and 1.3nm at 1 kV. It offers magnification from 25 to 1,000,000, an accelerating voltage range of 0.1kV to 30kV, and features detectors for both secondary and backscattered electrons. The eccentric specimen stage provides 5 axes motor control, including X-Y movement of 70mm×50mm, tilt from 5° to +70°, and 360° endless rotation. The preparation of samples followed the methodology outlined by Du et al. (2012). Initially, samples were separated and combined with the same volume of ethyl

acetate for a predetermined incubation period. The extracted product was then dried using anhydrous sodium sulfate (Na_2SO_4) in a rotary vacuum evaporator to remove any residual moisture. Subsequently, the dried samples were subjected to FESEM to visualize the surface morphology and obtain detailed high-resolution images of the surface characteristics.

5.8 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is an analytical method that analyses samples to determine their chemical properties using infrared light. This technique provides insight into the various functional groups existing in compounds within the sample (Kaur et al., 2021). The analysis was performed in Haryana Test House and Consultancy Services. The equipment utilized was the Perkin Elmer model, featuring KBr optics, a scan range of 8300-350 cm^{-1}, a resolution of0.5cm^{-1}, and a signal-to-noise ratio of 32,000:1. The ATR accessory used was diamond, and the analysis was facilitated using Spectrum 10 software. For FTIR analysis, the extracted product was examined, covering the mid-infrared range of 500–4500 cm^{-1} at a scanning speed of 16 min^{-1}. This technique identified and characterized the functional groups present on the surface of the samples, providing insights into the molecular bonds and structures within the sample (Vilchis-Carmona et al., 2021).

5.9 High-Performance Liquid Chromatography (HPLC)

HPLC is an effective analytical method used for qualitative, quantitative, and trace analysis of chemical and biological compounds. HPLC enables the precise determination of compound concentrations in a sample by measuring the height and area of chromatographic peaks (Ali, 2022). It is also adept at analyzing compounds present in very low concentrations. For dye de-colorization studies, reverse-phase HPLC was employed to analyze the metabolites resulting from the de-colorization process. After dye- dye-decolorization, the product was dissolved in (HPLC) grade carbinol and then passed through a C18 column (4.6 x 250 mm). A UV-Vis detector was part of the HPLC system, and the detection wavelengths (λ_{max}) were set to 465 nm for pure dye MO and 230 nm for the degraded dye sample. The procedure was carried o u t at room temperature (25°C).

The mobile phases, which consist of a 1:1 mixture of carbinol and water, were used to elute the samples through the column. Each sample was run for a total of 20 minutes, with the flow rates kept constant at 0.8 mL/min. This method allows for the effective separation and identification of the dye degradation products, providing crucial insights into the de-decolorization process and the efficiency of the microbial degradation mechanism (Islam et al., 2023).

By integrating high-resolution imaging capabilities of FESEM with molecular characterization provided by FTIR, a comprehensive understanding of surface properties and functional groups was achieved. Additionally, HPLC was employed to separate, identify, and quantify the various compounds involved in the process, elucidating the mechanisms underlying the microbial degradation and removal of Methyl Orange dye.

Objective 4: Toxicity analysis of the treated solution on selected agriculturally important crop seed germination.

5.10 Phytotoxicity and Microbial Toxicity Assessment

5.10.1 Phytotoxicity

The phytotoxicity study assessed the effects of Methyl Orange (MO) on ten agriculturally significant Indian crop varieties: *Cicer arietinum* (Chickpea), *Vigna radiata* (Mung bean), *Trigonella foenum-graecum* (Methi), *Brassica juncea* (Indian mustard), *Sorghum bicolor* (Jowar), *Vigna unguiculata* (Black-eyed pea or Lobia), *Hordeum vulgare* (Barley), *Phaseolus aconitifolius* (Moth Bean), *Vigna unguiculata* (Red Lobia), and *Zea mays* (Corn) (Yoon et al., 2015). Seeds were selected for uniformity, cleaned with distilled water, and surface-sterilized using 0.2% HgCl2 for one minute. The sterilized seeds were then placed on filter paper discs within Petri dishes and allowed to germinate for seven days at 37°C. Germination and subsequent growth measurements were conducted in triplicate, with ten seeds per replication (Bera et al., 2016). A 15 g/l concentration of MO was used for toxicity assessment, with the Control group treated with distilled water. The CCD was employed to treat the MO under optimal conditions, and evaluations of root lengths,

shoot lengths, and germination percentage was conducted after seven days (Watharkar et al., 2015).

5.9.2 Microbial Toxicity

To assess the microbial toxicity of MO dye post-removal by *Bacillus cereus* J4, standard Agar well diffusion methods were employed as described by Shah et al. (2013) and Dubey and Maheshwari (2002). This method involved measuring the microbial growth inhibition zones after a 24-hour incubation period at 35°C. The microorganisms used in this study included *Pseudomonas fluorescens* MTCC2421 and *Bacillus subtilis* MTCC441. The results provided insights into the potential toxicity of MO-treated effluents on microbial communities, which is crucial for understanding the broader environmental impacts of bio-remediated textile effluents.

Objective 5: Immobilization of the isolated strain for possible remediation of textile industry effluent.

5.10 Immobilization of *Bacillus cereus* J4 in Sodium Alginate Beads and De-colorization Processes

5.10.1 Process of Immobilizing Bacterial Strain in Sodium Alginate Beads

The immobilization of *Bacillus cereus* J4 cells in sodium alginate beads was performed following the methods described by Kar et al. (2008). Healthy *Bacillus cereus* J4 cells, harvested during the early log phase, were suspended in a sterile Na-alginate solution to achieve a density of 10^9 CFU cm^3. This suspension was extruded under constant agitation through a series of needles (0.2 mm diameter, 0.5 cm^3 min^{-1} rate) into a sterile 0.1 M $CaCl_2$ solution. The resulting beads were allowed to harden in the solution for ten minutes before being collected and rinsed with sterile distilled water. These alginate beads were subsequently used as packing material for both batch de-colorization and continuous flow column processes aimed at removing Methyl Orange (MO) from solutions.

FESEM: The surface morphology of sodium alginate beads, both those containing immobilized bacteria and those without, was meticulously analyzed before and after

undergoing dye adsorption treatments using a highly advanced JEOL USA Scanning Electron Microscope (Model JSM 6510LV). To prepare the samples for effective observation, they were first processed into a fine powder and then subjected to a vacuum drying process lasting 5 to 6 hours, ensuring the removal of any moisture. Once thoroughly dried, the samples underwent a gold sputtering process for 30 seconds with a DC magnetron sputtering machine, which provided a conductive coating essential for high-resolution imaging. Finally, the coated samples were carefully examined under the scanning electron microscopes at an accelerating voltage of 10 kV, allowing for detailed visualization of their surface characteristics and intricate interactions with the dye.

5.10.2 Batch De-colorization

For batch de-colorization, cell-immobilized beads were prepared by incorporating centrifuged bacterial cells into a hydrogel solution (Kar et al., 2008), which was then stirred for 30 minutes. Using a syringe, the mixture was injected into a cold 4% w/v CaCl2 solution to make spherical beads. After soaking in CaCl2 solution for a full day, the beads were stored at 4°C. In addition to live bacterial cells, the immobilization process was also performed with inactivated bacterial biomass. By autoclaving the bacteria for 15 minutes at 121°C, the bacteria became inactive. The inactivated cells were immobilized using the same procedure as live bacteria. (Hairunnisa FW et al., 2024).

5.10.3 Continuous De-colorization

The immobilized culture beads were then used as materials in a fixed-bed column made of borosilicate glass. The column had an internal diameter of 14 mm, a length of 236 mm, and a wall thickness of 2 mm. This setup was used to achieve continuous decolorization. The column was operated at two flow rates: 1.0 and 1.5 ml/min, regulated by a 20-liter capacity feed reservoir. Wastewater samples were collected after specified durations, and the remaining concentration of MO was calculated. The experimental setup included a schematic diagram of the column, illustrating the breakthrough curve, which represents the outcomes of the fixed-bed column (Figure 5.3). When the effluent (Ct) concentrations hit 50% influent concentrations (C0), a breakthrough happens. The column exhaustion point

occurs when the effluent concentrations become equal to the influent concentrations (90%). The breakthrough curve, typically plotted as Ct/C0 versus time or effluent volume, provides insights into the dynamic reactivity and properties of the column (Ahmad and Hameed, 2010). This methodology ensures an efficient and systematic approach to decolorizing Methyl Orange using immobilized Bacillus cereus J4, applicable in both batch and continuous flow systems.

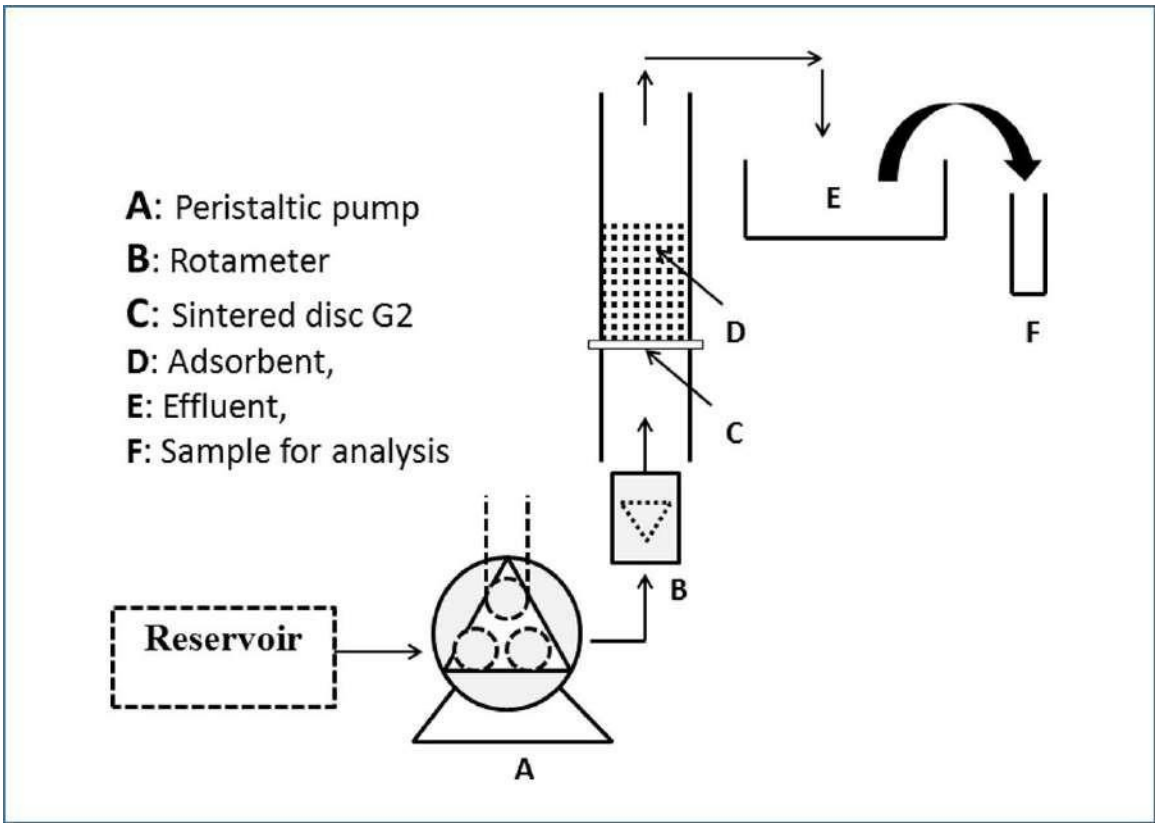


Figure 5.3: Diagram showing the fixed-bed column study's experimental setup

CHAPTER-6

RESULTS AND DISCUSSION

CHAPTER 6 – RESULTS AND DISCUSSION

Objective 1: Isolation of a new bacterial strain capable of removing methyl orange from aqueous solution and morphological identification, biochemical, and molecular characterization of the bacterial strain.

6.1 Isolation and screening of bacterial strains from textile industrial effluent and soils

In the present study, eleven morphologically distinct bacterial isolates designated J1 to J11 were obtained from soil samples enriched with textile industry effluent and wastewater. These isolates were screened for their efficiency in removing Methyl Orange (MO). Initially, isolates J4 and J10 demonstrated remarkable de-colorization potential and were selected for further experimental work. The isolates J4 and J10 were maintained on NA slants, and inoculum preparation followed the method described by Goswami et al. (2012). The isolation of different bacterial strains from these samples indicates their natural adaptations to survive in toxic dyes. This adaptation is due to their exposure to high dye concentrations and toxic effects, which is consistent with findings from previous studies (Khehra et al., 2005; Ali et al., 2009; Khaushik, 2009; Prasad and Rao, 2010; Pokhara and Ahluwalia, 2013; Dubey et al., 2010; Mahmood et al., 2011). The variation in de-colorization rates among the strains can be attributed to differences in specificity, structure, complexity, and positions of substituents in the aromatic rings, as well as their interaction with azo bonds in azo dyes (Carliell et al., 1995). Similar results were also observed by Saikia and Gopal (2004).

Further screening and biochemical testing identified isolate J4 as the most effective, achieving a maximum removal rate of 98% for Methyl Orange. Consequently, J4 was chosen for subsequent studies due to its superior de-colorization efficiency and application potential for textile wastewater bioremediation. This thorough selection process ensured that the most capable strain was identified for addressing the pollution and health hazards posed by textile dye effluents (Figure 6.1).

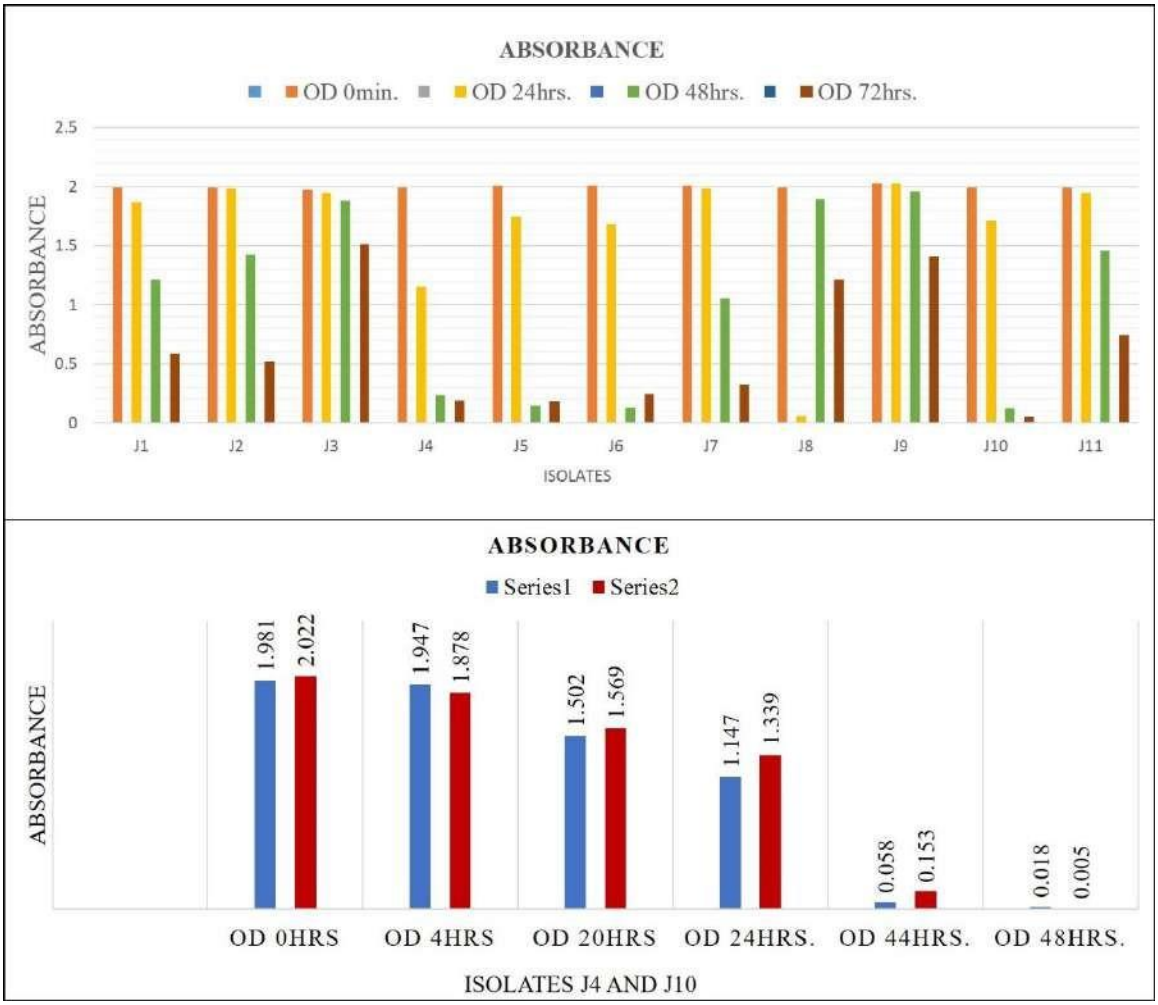


Figure 6.1: Absorbance of bacterial isolates, designated as J1 to J11, where J4 and J10 demonstrated remarkable de-colorization potential.

6.2 Morphological and Biochemical Identification

The identification of the bacterial isolates was conducted through physiological, morphological, and biochemical tests. The efficient bacterial strain J4 was selected for detailed characterization based on its cell morphology, surface and colony characteristics (color), and Gram's reaction. Gram stain characteristics indicated that J4 was a Gram-positive, rod-shaped bacterium. Morphologically, J4 displayed dull creamy colonies on nutrient agar plates and was small, rod-shaped, and non-motile (Figure 6.2).

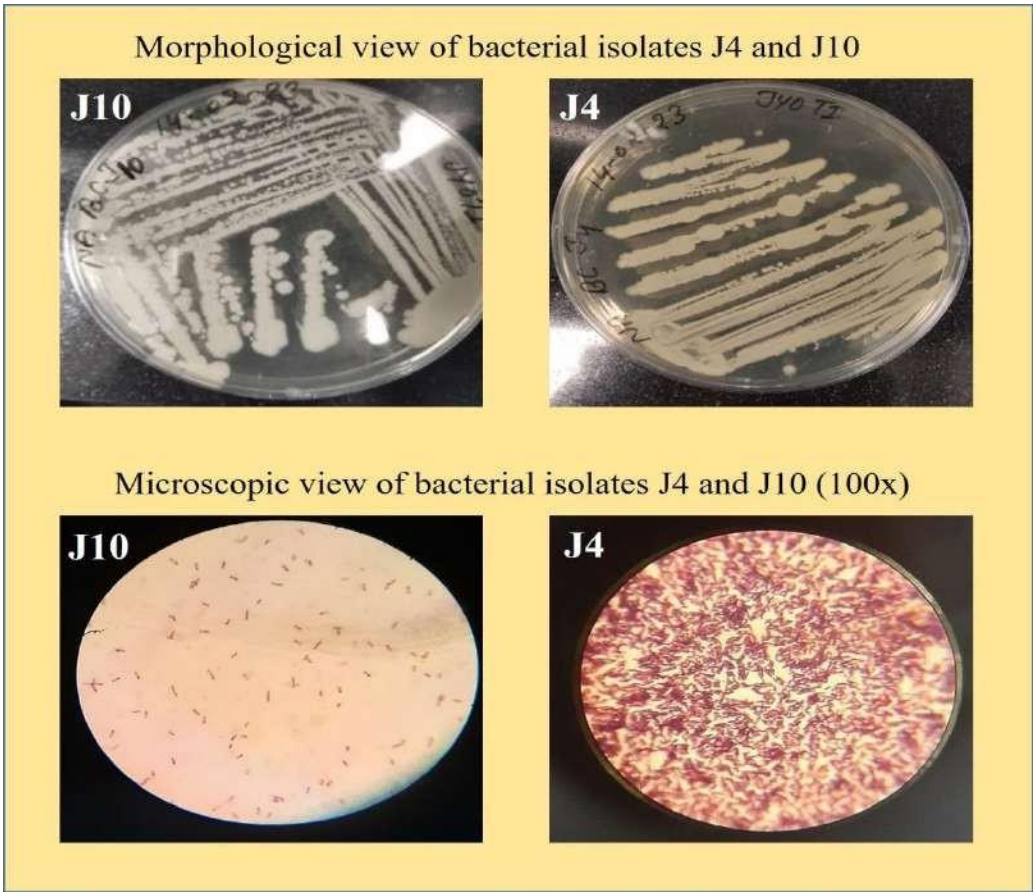


Figure 6.2: Morphological and microscopic (100X) view of J4 and J10 bacterial isolates.

The biochemical characterization of bacterial strains J4 and J10 revealed distinct profiles. Strain J4 exhibited negative results for the TSI slant, indole test, and citrate utilization while being non-motile. It showed positive results for urea utilization, lysine decarboxylation, and the (MR) test, but was negative for the (VP) test. Additionally, J4 was oxidase-positive and catalase-negative. Conversely, strain J10 also showed a negative TSI slant, indole test, and non-motility. However, J10 was negative for urea utilization, lysine decarboxylation, and the VP test, but it tested positive for citrate utilization and the MR test. Furthermore, J10 was both oxidase and catalase-positive. These biochemical tests help in differentiating the metabolic capabilities and enzymatic activities of the two bacterial strains, aiding in their identification and potential application in biodegradation processes (Table 6.1).

Table 6.1: Biochemical characterization of bacterial strains J4 and J10.

S.No.	Biochemical Tests	Typical Bacteria J4	Suspected Bacteria J10
1.	TSI Slant	-ve	-ve
2.	Motility Test	Non-motile	Non-motile
3.	Indole Test	-ve	-ve
4.	Urea utilization	+ve	-ve
5.	Lysine Decarboxylation	+ve	-ve
6.	Citrate Utilization	-ve	+ve
7.	Methyl Red	+ve	+ve
8.	Voges-Proskauer	-ve	-ve
9.	Oxidase reduction	+ve	+ve
10.	Catalase Test	-ve	+ve

To confirm the identity, 16S rRNA gene sequences were analyzed, and the sequences were subjected to a BLAST search and compared with the GenBank database. The phylogenetic analysis showed that J4 had a high degree of homology with Bacillus cereus (Figure 6.3). Consequently, strain J4 was identified as *Bacillus cereus* J4, with the sequence submitted to NCBI and assigned the accession number **OQ392442**. This comprehensive characterization confirmed the strain J4 as *Bacillus cereus* J4, highlighting its potential for applications in the bioremediation of Methyl Orange dye in textile effluents.

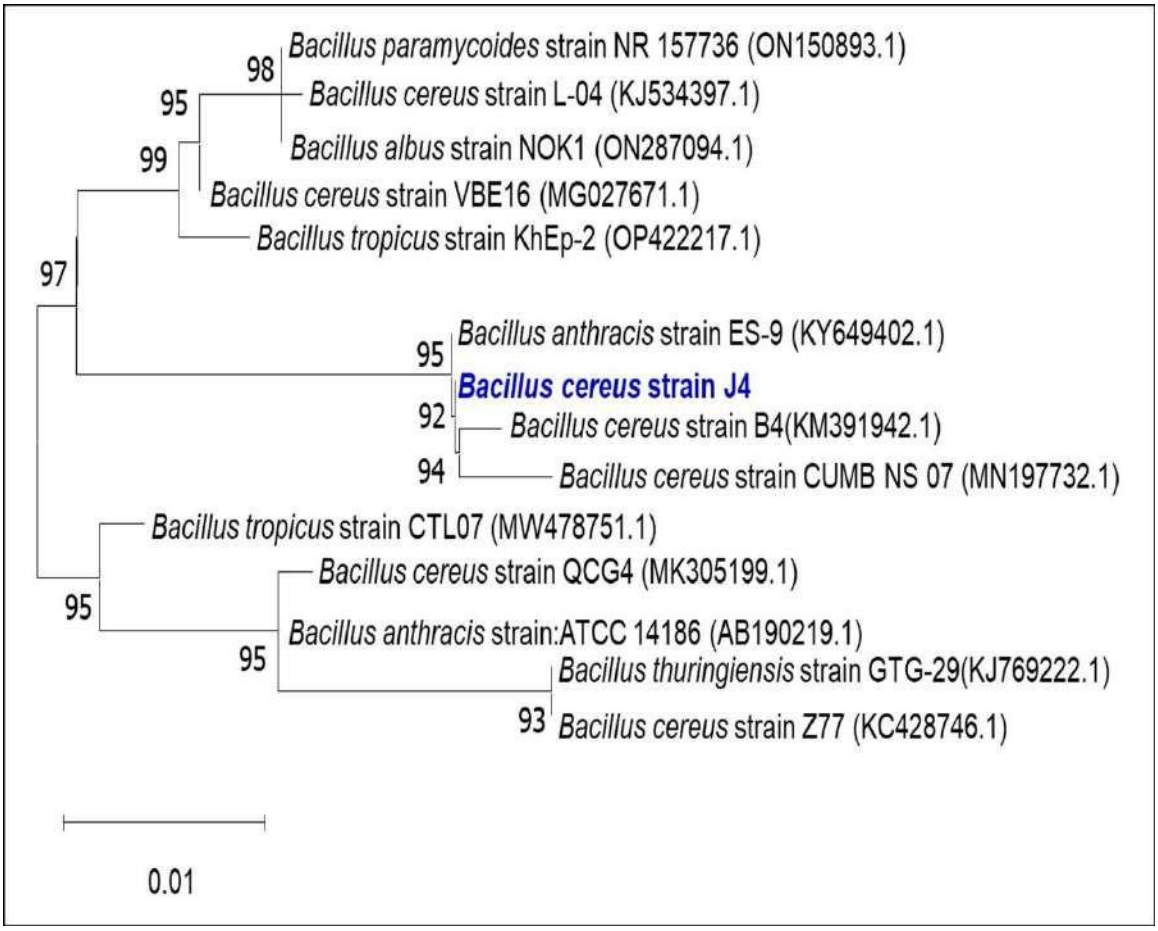


Figure 6.3: Phylogenetic tree of bacterium (*Bacillus cereus J4*).

Methyl Orange was fully decolorized by a unique strain of *Bacillus cereus* J4 after 48 hours of incubation, as confirmed by UV-visible spectroscopy (Figure 6.4). The primary mechanism behind this decolorization was degradation, rather than bio adsorption. The process of dye removal involved two subsequent stages: degradation and bio adsorption. During bio adsorption, the dye binds to the outer surface of bacterial cells, resulting in a colored biomass. However, degradation leads to the breakdown of the dye, leaving the cells colorless. After a 12-h incubation with Methyl Orange, methanol extracts of the cell pellets indicated that the dye had not been absorbed by the cell biomass. This observation supports the conclusion that the decolorization of MO by *Bacillus cereus* J4 was primarily due to the breakdown of the dye rather than its adsorption onto the bacterial cells.

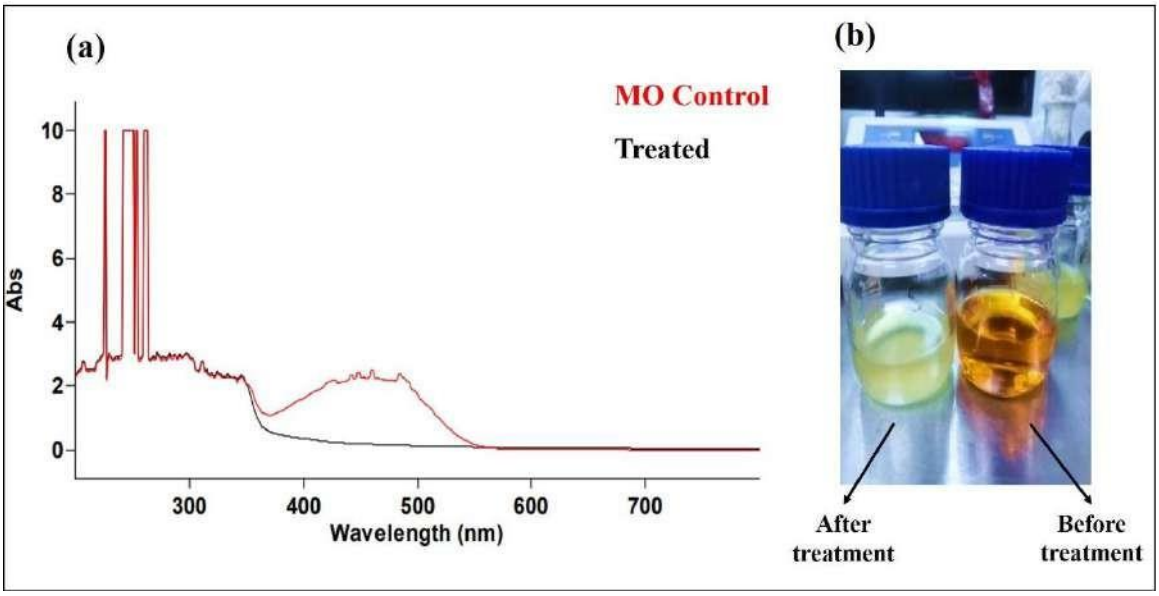


Figure 6.4: (a) UV spectra of MO, (b) before treatments and after treatments.

Objective 2: Statistical optimization of the Physico-chemical parameters for rapid removal of MO.

6.3 Optimization of physical-chemical parameters (OVAT experiments)

Optimization of culture conditions was required to enhance the dye de-colorization activity. Azo dye de-colorization is highly influenced by physical factors (Initial Dye concentration, Inoculum Percentage, Incubation Time, pH, Temperature, and Agitation speed) and chemical factors (extra carbon and extra nitrogen sources). These physical-chemical parameters are important for bacterial growth and dye removal.

6.3.1 Effects of Initial Dye Concentrations on Dye Removal %

The maximum decolorization ability of Bacillus cereus J4 was achieved by optimizing various physicochemical parameters, including initial dye concentrations ranging from 10 to 100 mg/L. The decolorization percentages were found to be inversely proportional to dye concentrations, with higher concentrations leading to increased toxicity and subsequent suppression of cellular activities (Shah et. al, 2013a). For industrial applications, it is crucial to identify microorganisms capable of degrading high

concentrations of dyes. Industrial effluents typically contain dye concentrations between 10 and 50 mg/L (Padamavathy et al., 2003). *Bacillus cereus* J4 demonstrated the potential to effectively remove methyl orange up to 40 mg/l, indicating its suitability for practical use in treating textile wastewater. This confirms that the selected strain can achieve significant de-colorization, making it a viable candidate for bioremediation efforts.

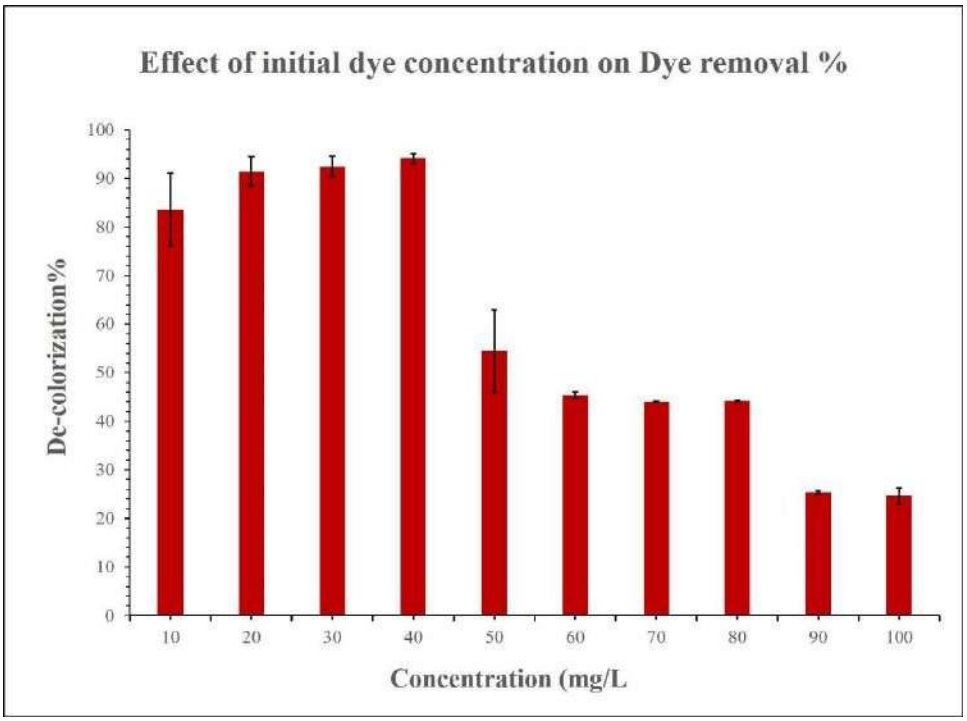


Figure 6.5: Effects of initial dye concentration on de-colorization%

6.3.2 Effects of inoculum percentage on dye removal %

To assess the effect of inoculum percentage on dye removal, 250 ml flasks were inoculated with varying concentrations of the bacterial culture (0.5%, 1%, 1.5%, 2%, 2.5%) and added with 50 mg/l of dye solution at the optimal concentration, with each flask containing 50 ml of NB. These were incubated at the ideal temperature. The decolorization percentage in each sample was determined by taking the aliquots at specific time intervals, centrifuging them, and measuring the extent of dye removal. The level of decolorization experienced a threefold increase, rising from 0.5% to 1.5%, ultimately achieving the highest decolorization of MO at 1.5% (6.6). A higher percentage of decolorization may have

resulted from an increased surface area for dye sorption caused by a larger amount of inoculum. This method allowed for the determination of the most effective inoculum percentage for maximizing dye de-colorization efficiency.

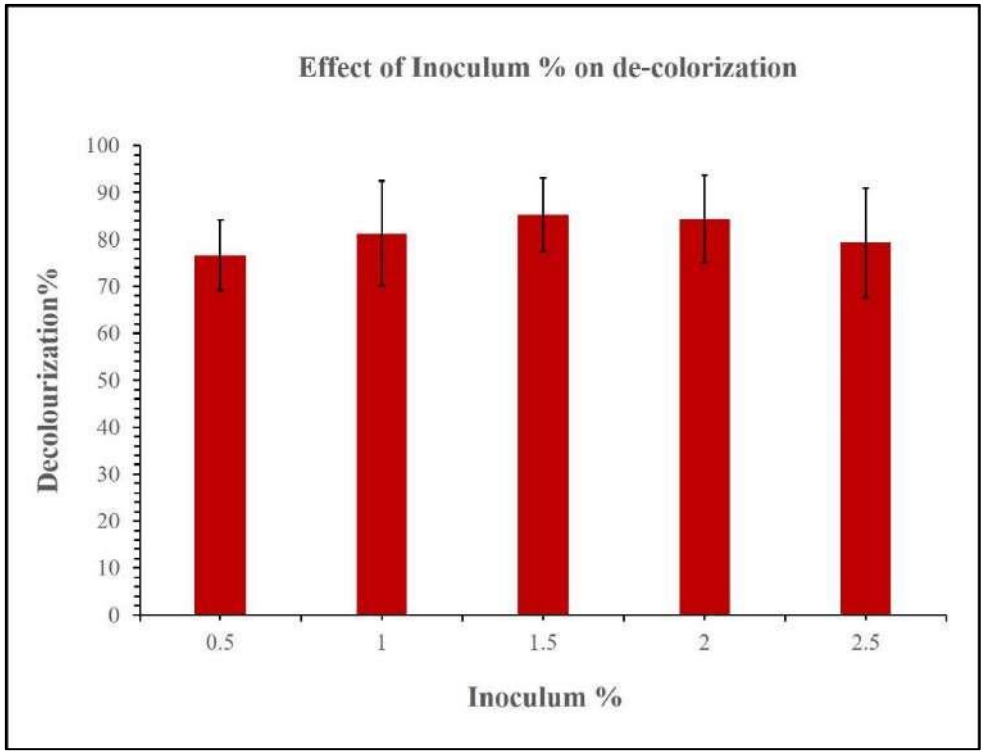


Figure 6.6: Effect of inoculum percentage on de-colorization%

6.3.3 Effects of incubation time on de-colorization %

To investigate the impact of different incubation times on the efficiency of dye decolorization, 250 ml flasks with 50 ml of NB and 50 mg/l of dye solutions were inoculated with fresh bacterial cultures and then placed in an incubator at a temperature of 37°C. Aliquots of the culture media were withdrawn at different time intervals (8, 16, 24, 32, 40, and 48 hours), centrifuged10,000 rpm, and the supernatent was analyzed for de-colorization using a spectrophotometer. The percentages of de-colorization were calculated based on these measurements. The strain exhibited 53% de-colorization of Methyl Orange within the first eight hours of incubation. Following extended incubation, bacterial strain J4

achieved a maximum de-colorization of 96% after 40 hours, demonstrating its high efficiency in degrading the dye over time.

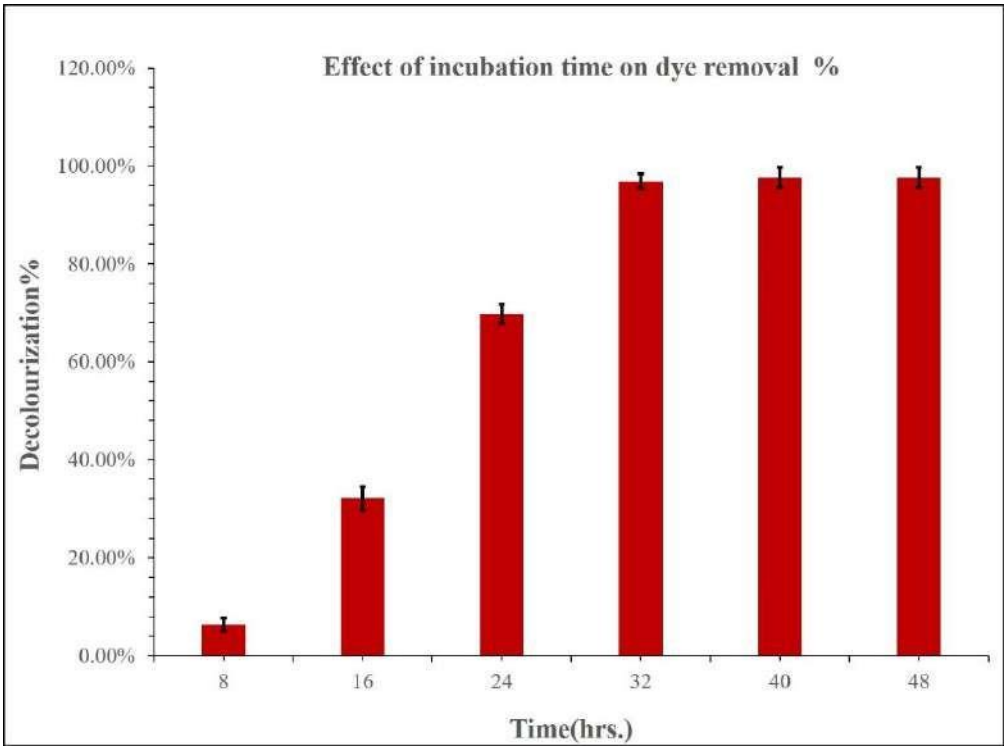


Figure 6.7: Effects of incubation time on dye removal %

6.3.4 Effects of temperature on dyes removal %

In assessing the de-colorization efficiency of *Bacillus* sp. under different temperature conditions, an investigation was conducted to find the optimal temperature for maximizing dye removal. On the fifth day of incubation, it was observed that *Bacillus* sp. exhibited the highest de-colorization percentage (64.34%) at the optimal temperature of 37°C. This efficiency was compared to other temperature ranges (25°C to 50°C), where lower de-colorization percentages were noted. This indicates that 37°C is the ideal temperature for maximum de-colorization activity by *Bacillus* sp.

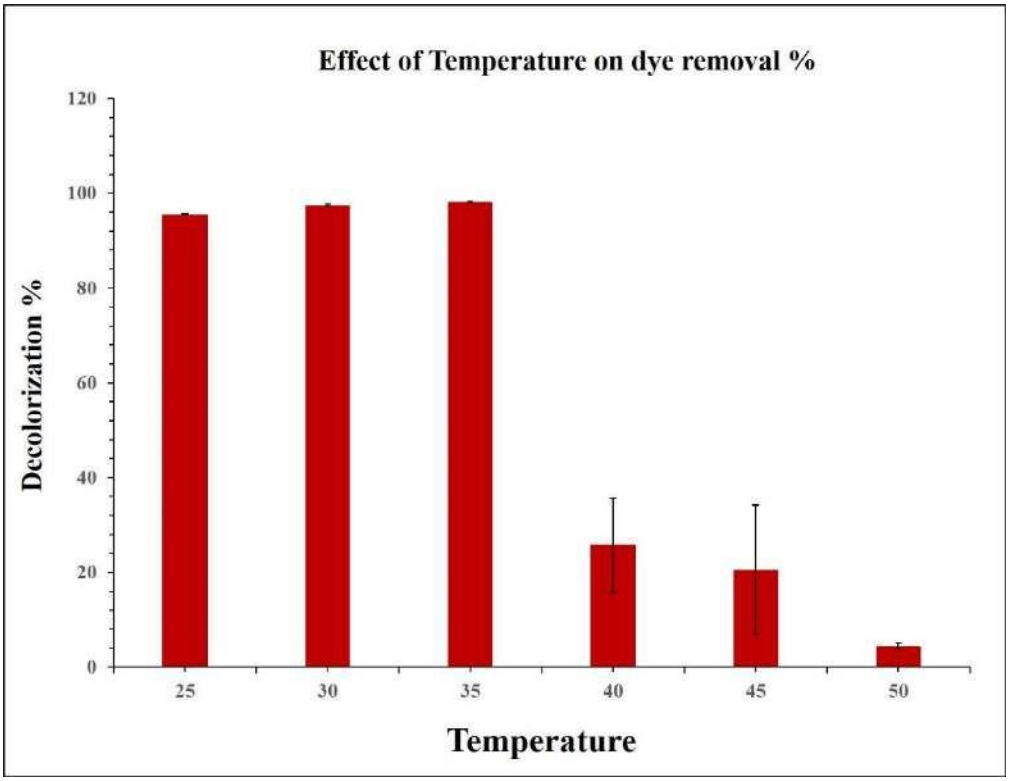


Figure 6.8: Effects of temperature on de-colorization%

6.3.5 Effects of pH on de-colorization%

An investigation was undertaken to assess the impact of pH on the removal efficiency of MO by *Bacillus cereus* J4 across a pH range of 4 to 10. Results revealed an increase in de-colorization from pH 4 to 8, with optimal de-colorization observed at pH 8. Beyond pH 8 and below pH 4, the de-colorization efficacy of *Bacillus cereus* J4 was notably reduced. This finding is consistent with other research, suggesting that pH plays a crucial role in removing dye particles through cell membranes, acting as a limiting factor for de-colorization. Specifically, at acidic pH (<4), the presence of H^+ ions inhibits the de-colorization process, while at higher pH (>4), the negatively charged biomass surface attracts positively charged dye cations electrostatically, enhancing de-colorization efficiency. These findings corroborate earlier research outcomes, providing valuable insights into the pH-dependent de-colorization behavior of methyl orange by *Bacillus cereus* J4.

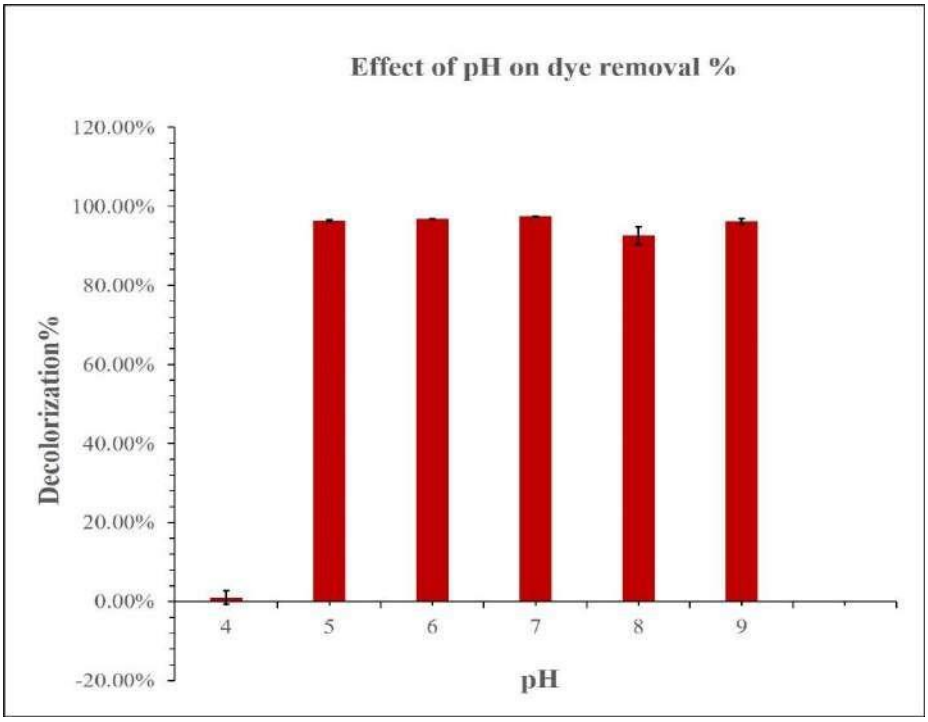


Figure 6.9: Effects of pH on de-colorization%

6.3.6 Effects of agitation speeds on de-colorization%

The influence of agitation speeds on the removal efficacy of Methyl Orange (MO) by *Bacillus cereus* J4 was examined in this study. Several agitation speeds (RPM) were tested to determine their effect on MO removal under strict aerobic conditions. Results demonstrated effective MO removal, with the optimal removal rate achieved at 120 RPM, within the range of 0 to 200 RPM. However, agitation speeds exceeding 200 RPM led to a reduction in the dye removal rate, potentially due to the heightened aeration rate inducing toxic effects on *Bacillus cereus* J4. This observation underscores the importance of environmental conditions in influencing the dye de-colorization process, as environmental factors can directly impact microbial metabolism, thereby affecting the efficiency of dye removal. These findings contribute valuable insights into optimizing agitation speed for enhanced MO removal by *Bacillus cereus* J4

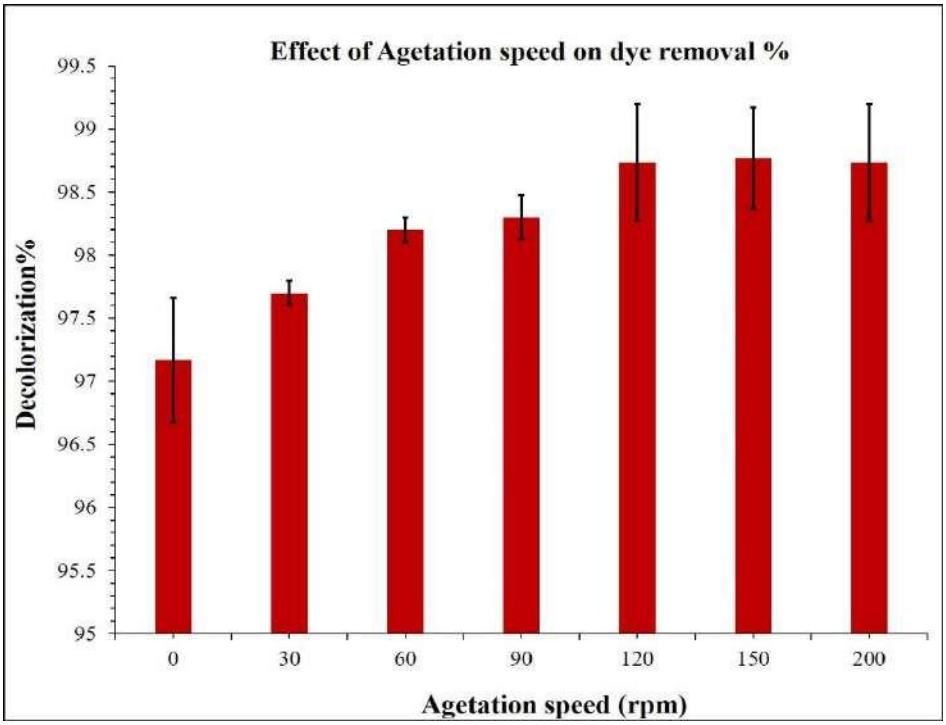
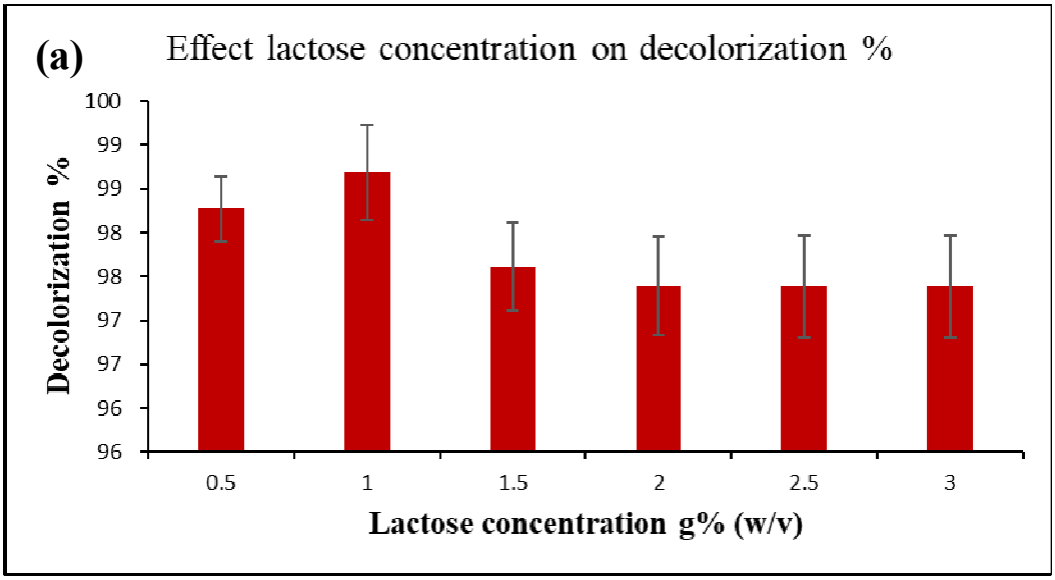


Figure 6.10: Effects of agitation speed on de-colorization%

6.3.7 Effects of Extra Carbon Sources on de-colorization%

In consideration of the intricate molecular structure of dyes, only a limited number of microorganisms possess the capability to utilize dyes as their primary carbon source (Das and Mishra, 2017). The de-colorization process may be hindered by the depletion of critical nutrient molecules during bacterial growth. To explore the role of different carbon sources in Methyl Orange dye de-colorization, glucose, sucrose, fructose, mannitol, and lactose were supplemented in the culture medium. Among these sources, lactose exhibited the highest dye removal efficiency at 98.71%, followed by fructose at 96.12%. Further optimization of lactose concentration, ranging from 0.5 to 3 % (w/v), revealed that a concentration of 1 % (w/v) yielded the highest de-colorization percentage of 98.86%, surpassing other concentrations of carbon sources (Figure 6.11a). Consequently, lactose at a concentration of 1 % (w/v) emerged as the optimal carbon source for Methyl Orange removal using *Bacillus cereus* J4 (Figure 6.11b). These findings underscore the

significance of lactose as an effective carbon source for enhancing the de-colorization efficiency of Methyl Orange by *Bacillus cereus* J4.



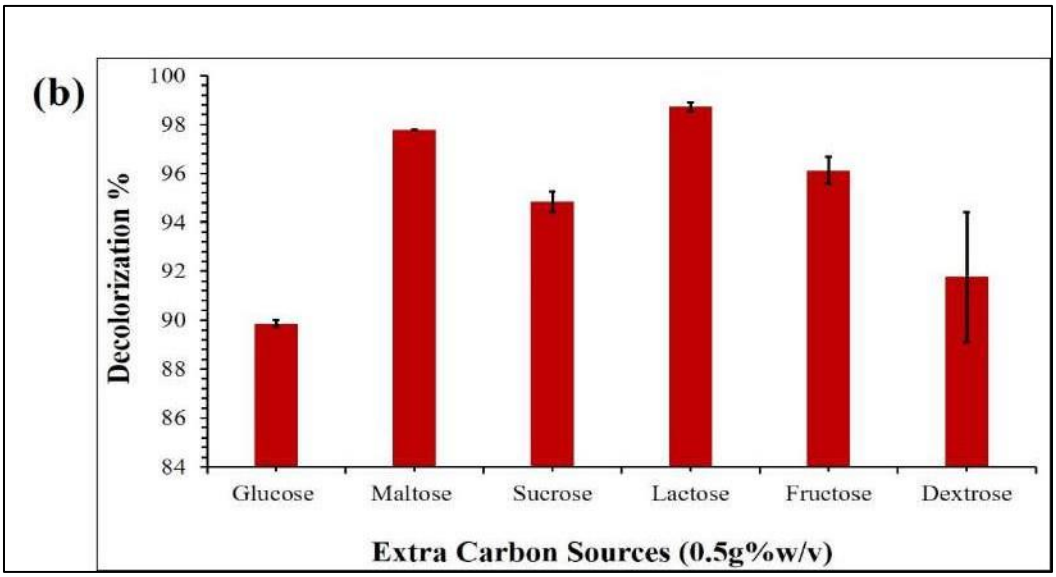


Figure 6.11: (a) Effects of lactose concentrations % (w/v), (b) Effects of Extra carbon sources (Extra carbon) on decolorization%

6.3.8 Effect of Extra Nitrogen Source on decolorization%

Yeast extract has traditionally served as a prevalent nitrogen source in many de-colorization processes involving bacteria (Das and Mishra, 2017). Nevertheless, to evaluate alternative nitrogen sources for Methyl Orange dye removal, sodium nitrite, ammonium sulfate, ammonium nitrate, L-tryptophan, peptone, and urea were introduced into the culture medium. Results demonstrated that all nitrogen sources, except sodium nitrite and ammonium nitrate, yielded de-colorization percentages exceeding 80% (Figure 6.12a). Among these options, urea emerged as particularly promising due to its cost-effectiveness and widespread availability in agricultural settings as residual material. Following the identification of urea as the optimal nitrogen source, the next step involved optimizing its concentration. As depicted in Figure 6.12(b), the optimization process involved varying urea concentrations from 0.5 to 3 % (w/v). The graphical representation of the data indicated that a concentration of 2 % (w/v) of urea achieved the highest decolorization efficiency of the azo dye (MO). These findings underscore the significance of urea as a viable nitrogen source for enhancing the de-colorization process of Methyl Orange, highlighting its potential for practical application in wastewater treatment processes.

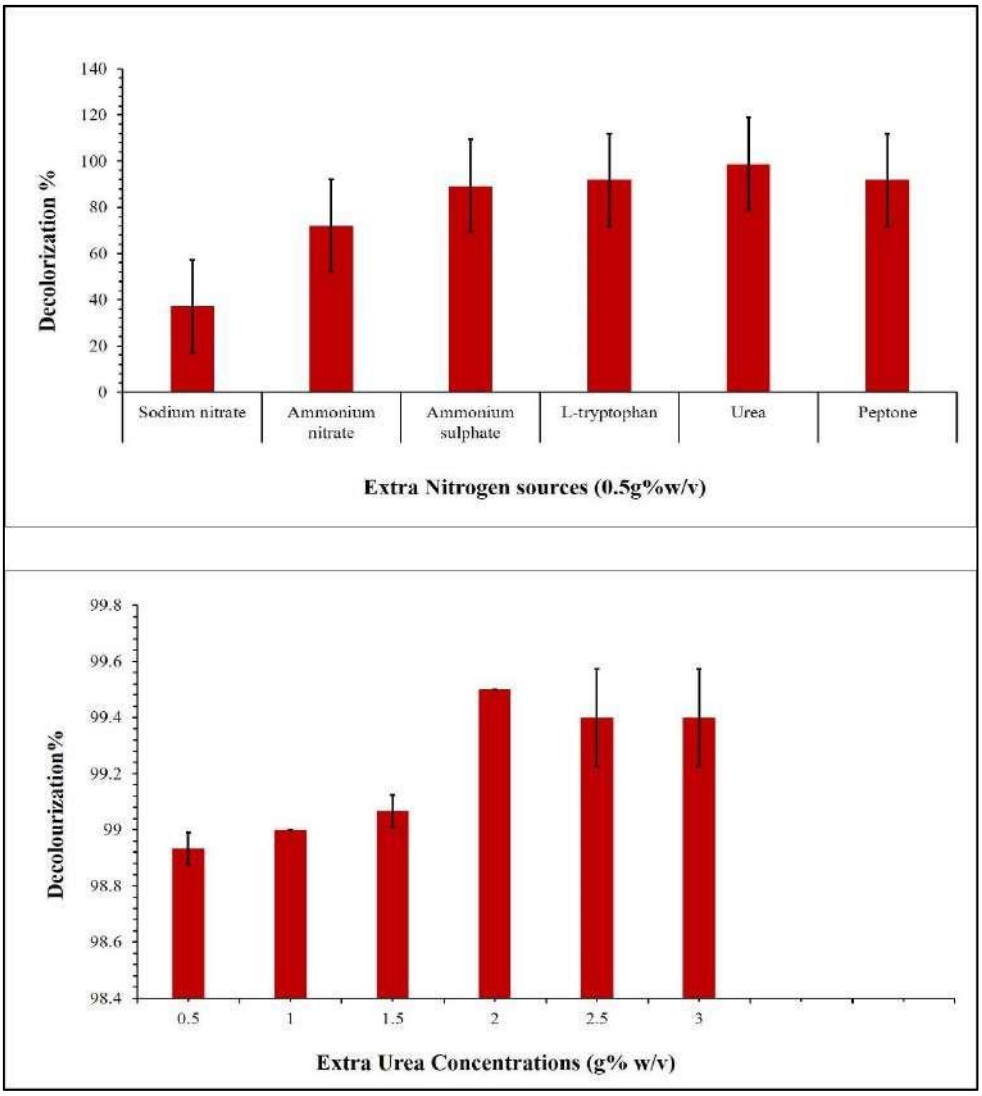


Figure 6.12: (a) Effect of Extra Nitrogen Sources on decolorization % (b) Effect of Urea concentration % w/v on de-colorization%.

The de-colorization of the azo dye (MO) is significantly influenced by various factors, including initial dye concentration, inoculum percentage, incubation time, temperature, pH, agitation speed, extra carbon, and extra nitrogen sources. The current studies highlight the impact of these variables on the effectiveness of bacterial decolorization. For instance, it was found that varying the initial concentration of MO from 10-50 mg/L influences the microbial degradation rate, with lower concentrations facilitating more efficient de-colorization (Singh et. al., 2022). Likewise, Kumar et. al. (2023) found that an inoculum

size of 1-2% (v/v) is optimal for microbial activity. Another study demonstrated that a 48-hour incubation period is ideal for achieving maximum de-colorization by *Bacillus cereus* (Sharma et al., 2022). The pH and temperature of the culture medium, ideally neutral to slightly alkaline (pH 7-8) and within 30-37°C, were shown to significantly impact microbial metabolism and enzyme activity (Zhang et al., 2023). Proper aeration and mixing, achieved with an agitation speed of 150 rpm, are essential for ensuring uniform exposure of the microbes to the dye (Lee et al., 2023). Additionally, supplementing the medium with extra carbon and nitrogen sources, such as glucose or peptone, can significantly improve decolorization rates (Patel et al., 2022). Each microbial strain has unique requirements, and adjusting the medium composition to meet these specific needs is vital for enhancing dye degradation efficiency (Hassan et al., 2022). Thus, a customized approach to optimize both physical and chemical parameters is necessary to achieve efficient and effective dye de-colorization, contributing to sustainable wastewater treatment solutions.

6.4 Statistical optimization of physicochemical parameters

The assessment of methyl orange removal was conducted using the Plackett-Burman (PB) method to screen for influential factors. In PB design, the primary impacts are often obscured by interactions between two factors. Pareto chart analysis of PB results concerning physical and chemical factors revealed that the initial concentration (A), initial pH (E), temperature (D), and nitrogen source (H) exerted the most significant influence on the maximum de-colorization of Methyl Orange dye (MO). Furthermore, a notable p-value (<0.05) underscored the significance of these factors in dye removal. The entire model for the PB design was deemed significant, as indicated by a number bigger than four, signifying satisfactory signal strength for the system. These findings highlight the critical role of initial concentration, pH, temperature, and nitrogen source in optimizing the removal of Methyl Orange dye, providing valuable insights for process optimization in wastewater treatment applications.

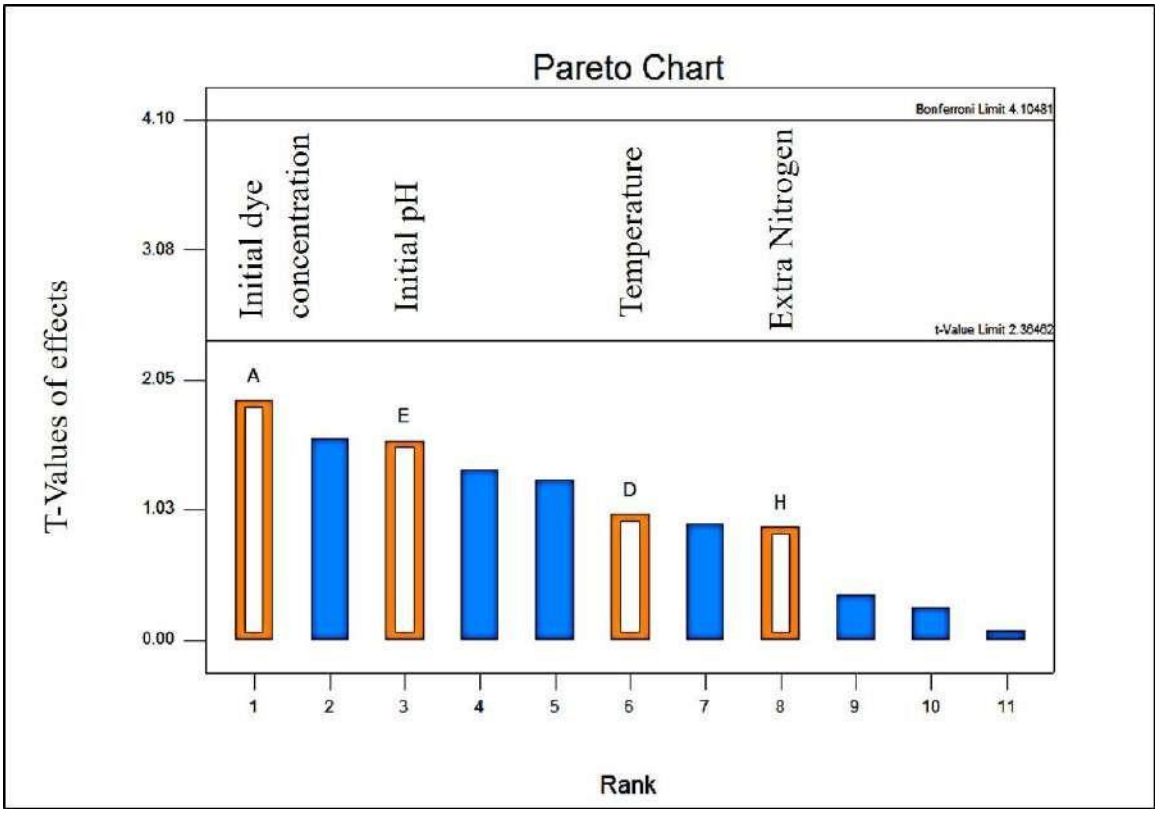


Figure 6.13: Pareto Chart analysis of Plackett-Burman results.

6.5 Statistical optimization using RSM

RSM and CCD were utilized to evaluate the impacts of four important variables on removing (MO) methyl orange. While initial dye concentration, pH, temperature, and additional nitrogen source exhibited significant positive p-values in the preliminary Plackett-Burman (PB) design, they were omitted from the CCD design due to cost and power consumption constraints associated with industrial-scale wastewater treatment. Generated experiments, listed in Table 6.2, were employed to determine dye removal percentages for each experiment involving methyl orange de-colorization. Regression analysis, employing a quadratic model ($p< 0.001$), was utilized to fit the responses, with a square root conversion yielding R2 = 0.9686, R2 Adj = 0.9393, R2 Pred = 0.8194, and an adequate precision of 16.96. Coefficients (R2) and adjusted R2 (R2 Adj) were computed to assess model suitability, with satisfactory values of R2 (0.9894) and R2 Adj (0.9393)

obtained. Temperature and pH had the second most significant connection ($p < 0.0094$) in methyl orange decolorization, after the combined effects of pH and starting dye concentration ($p < 0.0012$) in ANOVA (Table 6.3). While the significance value of the nitrogen source and initial dye concentration was comparatively low, the overall model remained significant ($p < 0.0001$). Each factor was determined to be significant ($p < 0.0001$), with initial pH exerting the most influence ($p < 0.0007$) on the de-colorization process (F value = 18.29).

Table 6.2: Experimental runs for the de-coloization of dye MO using CCD

Run	**Initial dye concentration (A)**	**pH (B)**	**Temperature (C)**	**Extra nitrogen source (Urea) (D)**	**Dye removal (%)**
1.	55.00	6.50	37.50	-1.50	99.23
2.	55.00	6.50	12.50	1.50	2.11
3.	10.00	400	50.00	0.00	12.12
4.	55.00	6.50	37.50	4.50	98.04
5.	55.00	6.50	37.50	1.50	99.23
6.	100.00	4.00	50.00	3.00	10.24
7.	100.00	4.00	50.00	0.00	20.11
8.	55.00	6.50	37.50	1.50	99.32
9.	100.00	9.00	25.00	0.00	97.21
10.	55.00	6.50	37.50	1.50	98.27
11.	55.00	6.50	37.50	1.50	99.76
12.	100.00	9.00	25.00	3.00	50.23
13.	145.00	6.50	37.50	1.50	1.11
14.	55.00	1.50	37.50	1.50	1.22
15.	10.00	9.00	25.00	3.00	22.21
16.	55.00	6.50	37.50	1.50	99.87
17.	55.00	11.50	37.50	1.50	20.23

18.	10.00	9.00	50.00	3.00	1.14
19.	10.00	4.00	25.00	3.00	12.15
20.	100.00	4.00	25.00	3.00	1.14
21.	10.00	4.00	25.00	0.00	22.32
22.	10.00	4.00	50.00	3.00	15.11
23.	-35.00	6.50	37.50	1.50	1.02
24.	100.00	9.00	50.00	0.00	43.24
25.	55.00	6.50	37.50	1.50	99.78
26.	10.00	9.00	50.00	0.00	11.18
27.	100.00	9.00	50.00	3.00	28.17
28.	100.00	4.00	25.00	0.00	22.31
29.	55.00	6.50	62.50	1.50	1.02
30.	10.00	9.00	25.00	0.00	33.43

The study investigated the effects of various factors on the de-colorization of MO by *Bacillus cereus* J4, with results illustrated in Figure 6.14 (a), 6.14 (b), 6.14 (c), 6.14 (d), and 6.14 (e) through 3-D responses and contour diagrams, while Figure 6.18 illustrates the predicted versus actual values plot.

Figure 6.14 (a) presents the impact of pH and initial dye concentration on MO removal. At low initial dye concentrations (10 ppm) and acidic pH (4), the removal efficiency was approximately 39%. Increasing the dye concentration showed a marginal increase in removal efficiency to 40.3% at the same pH level. Notably, when the pH was increased towards alkaline conditions, the de-colorization efficiency significantly improved, reaching approximately 85.5%. This suggests that neutral to slightly alkaline pH provides optimal conditions for bacterial activity and MO de-colorization, consistent with previous findings that bacterial physiology and enzyme activities are highly pH-dependent (Chang and Lin, 2001).

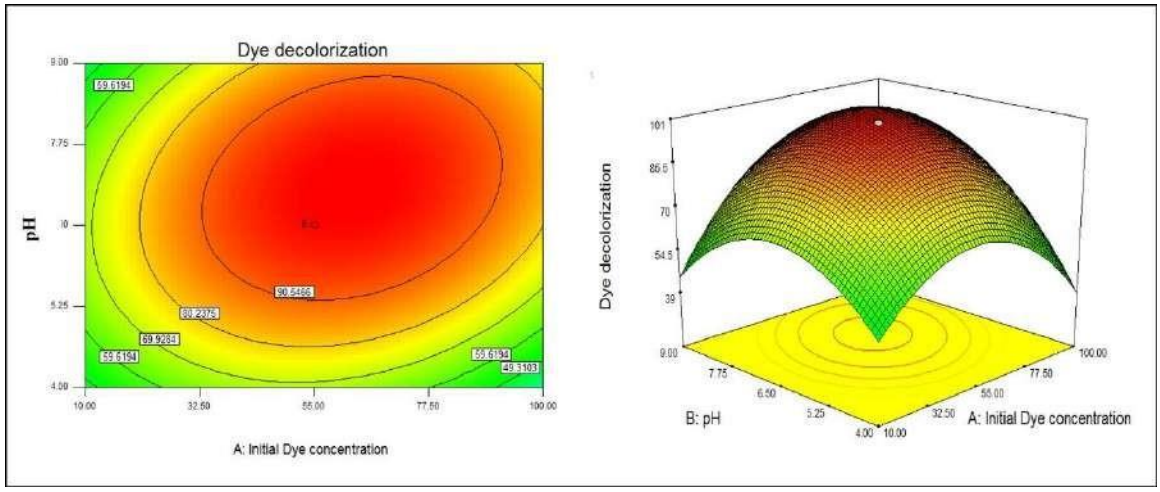


Figure 6.14 (a): Contour plots correlated to response surface (3D) plots for the interaction of pH vs Initial dye for the de-colorization of Methyl Orange using *Bacillus cereus J4.*

Figure 6.14 (b) shows the relationship between temperature and initial dye concentration. At lower temperatures (25–30°C) and 10 ppm dye concentration, the removal efficiency was less than 39%. At a dye concentration of 55 ppm and a temperature of 37.5°C, the removal efficiency increased to 89.5%. This trend underscores the importance of maintaining an optimal temperature for bacterial growth and activity, as supported by research indicating that extreme temperatures can inhibit microbial metabolism (Shah et al., 2013a).

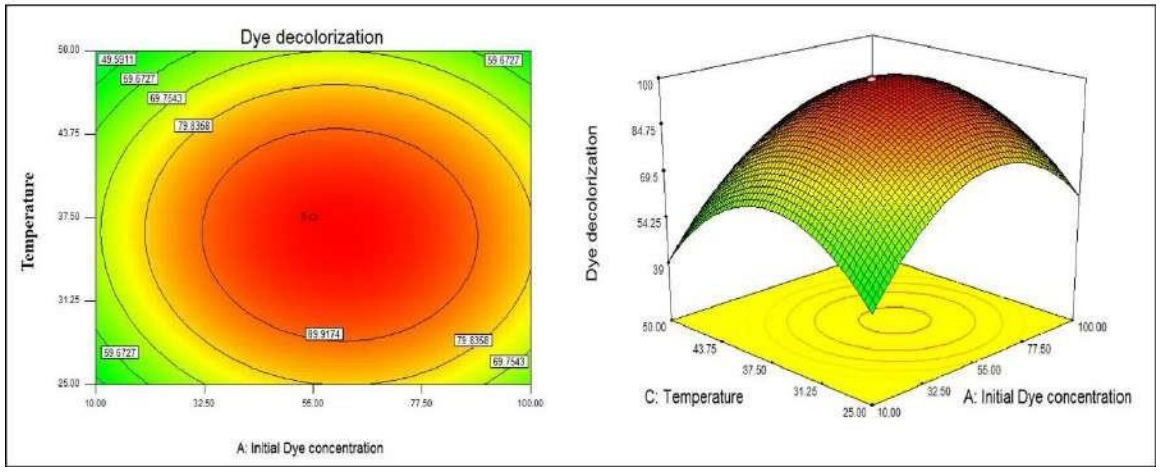


Figure 6.14 (b): Contour plots correlated to response surface (3D) plots for the interaction of temperature vs Initial dye for the de-colorization of Methyl Orange using *Bacillus cereus J4.*

Figure 6.14 (c) illustrates the effect of urea concentration on MO removal under constant initial dye concentration. An increase in nitrogen concentration from 0.5% to 3% led to a nearly 10% enhancement in MO removal, from 71% to 81%. This indicates that additional nitrogen sources such as urea can significantly boost bacterial metabolic activity and improve dye de-colorization efficiency, supporting findings by Daneshvar et al. (2007) on the role of nitrogen in microbial de-colorization processes.

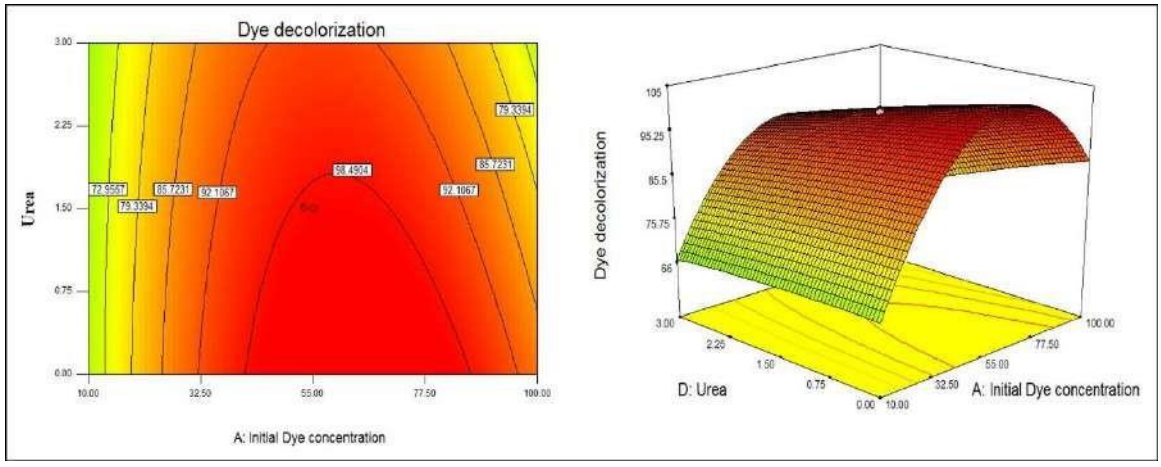


Figure 6.14 (c): Contour plots correlated to response surface (3D) plots for the interaction of urea vs Initial dye for the de-colorization of Methyl Orange using *Bacillus cereus J4.*

Figure 6.14 (d) explores the interaction between pH and temperature on MO removal. The combined effect of pH and temperature did not show a significant difference in removal efficiency, although the optimal temperature of 37°C at varying pH levels enhanced de-colorization. This aligns with studies highlighting that while individual parameters are crucial, their interactions can have complex effects on microbial processes (Bardi and Marzona, 2010).

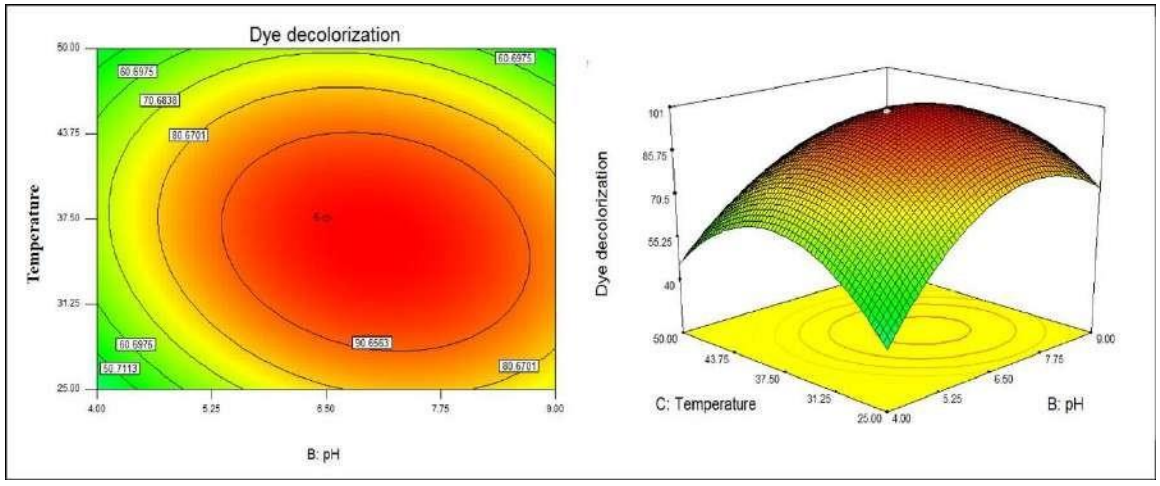


Figure 6.14 (d): Contour plots correlated to response surface (3D) plots for the interaction of temperature vs pH for the de-colorization of Methyl Orange using *Bacillus cereus J4.*

Figure 6.14 (e) examines the combined impact of urea and temperature on MO removal. A significant improvement in de-colorization was observed at 37°C with increased urea concentration, indicating that urea acts as an effective enhancer of bacterial de-colorization activity. This supports the notion that nutrient supplementation can optimize microbial dye degradation processes (Das and Mishra, 2017).

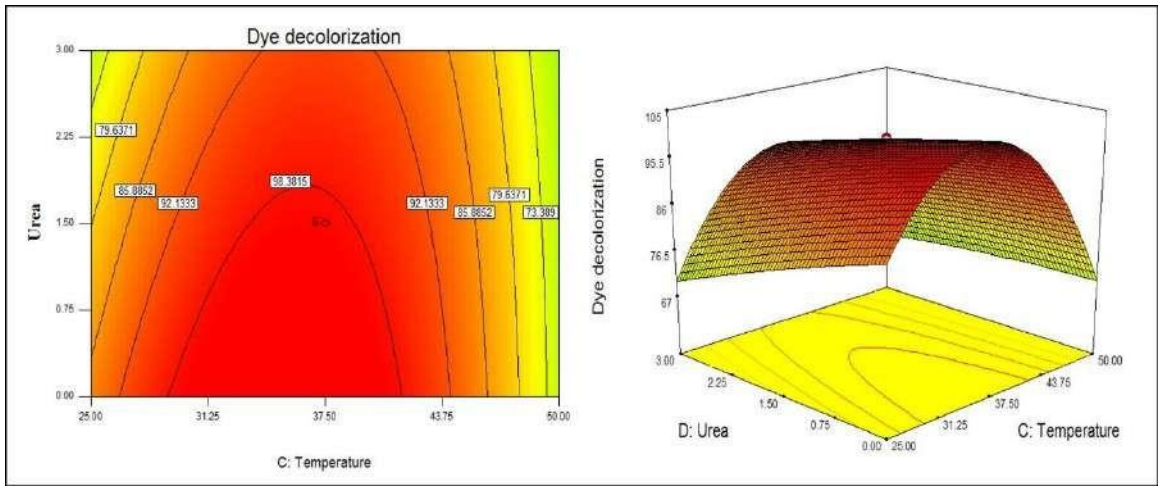


Figure 6.14 (e): Contour plots correlated to response surface (3D) plots for the interaction of urea vs temperature for the de-colorization of Methyl Orange using *Bacillus cereus J4.*

Overall, the study revealed that initial dye concentration, pH, temperature, and additional nitrogen sources are key factors influencing the de-colorization of Methyl Orange by *Bacillus cereus* J4. The findings highlight the necessity of optimizing these parameters to achieve maximum dye removal, emphasizing the strain's potential for efficient and cost-effective wastewater treatment. These results align with previous research on microbial de-colorization, suggesting that adjusted environmental conditions are essential for enhancing bioremediation processes (Shobana and Hangam, 2012)

Initial pH, temperature, and additional nitrogen were identified as crucial factors for optimizing the MO dye removal. The ANOVA analysis (Table 6.3) (Figure 6.15) indicated that the optimal model for MO removal was achieved with conditions of 55 ppm initial dye concentration, pH 6.50, 37°C, and 1.5% urea as an additional nitrogen source. Under these conditions, the model demonstrated an 88.93% removal efficacy of methyl Orange (MO) from an aqueous solution, with a desirability value of 1. The practical efficacy of this model was further confirmed by a 93.05% dye removal rate observed in validation experiments. Using the results from the (RSM), the optimized conditions was predicted and confirmed through triplicate experiments, underscoring the model's reliability (Table 6.4). From the textile industry sector of Panipat, Haryana, India, eleven bacterial strains were found for this study. *Bacillus cereus* J4 showed the most potential of them, being able to extract 89.8% of Methyl Orange from aqueous solutions. This high efficiency is particularly notable as it positions *Bacillus cereus* J4 as a promising agent for batch removal processes of MO. Several physical and chemical parameters significantly influence the rate of MO de-colorization. According to statistical analysis, the initial dye concentration, temperature, additional nitrogen sources (urea), and pH are the key variables impacting MO de-colorization.

The significance of these factors is supported by previous research. For example, Chang and Lin (2001) highlighted that the crucial role pH plays in enzymatic activity and microbial physiology is in line with our finding that dye removal is most effective at a pH of 6.50. Similarly, Das and Mishra (2017) emphasized the importance of nutrient supplementation, which aligns with our observation that urea enhances bacterial activity

and dye removal efficiency. Temperature optimization, crucial for microbial growth and dye de-colorization, is consistent with the findings of Bardi and Marzona (2010), who reported that extreme temperatures inhibit microbial activity. Therefore, *Bacillus cereus* J4 is a valuable agent for the safe release of MO from industrial effluents into the environment. By optimizing the initial dye concentration, pH, temperature, and additional nitrogen sources, this bacterium can greatly enhance the efficiency of MO de-colorization, offering a viable and cost-effective solution for wastewater treatment in industrial applications.

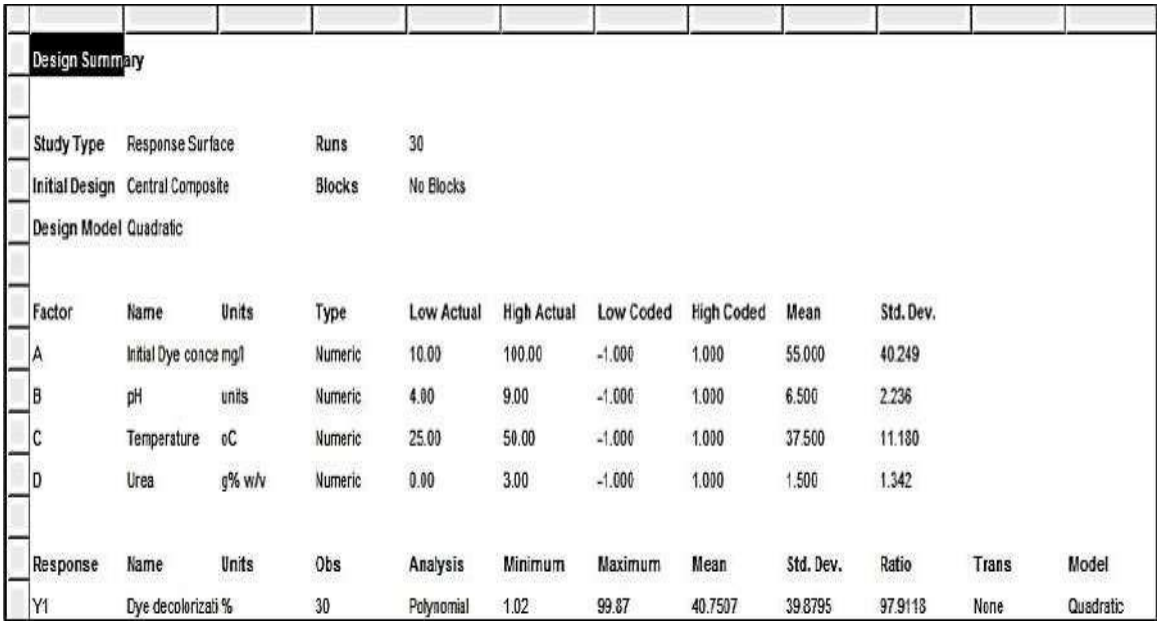
Table 6.3: ANOVA Table

Source	**Sum of squares**	**Degree of freedom**	**Mean Squares**	**F-value**	**p-Value Prob >F**	
Model	46213.62	14	3300.97	33.06	<0.0001	Significant
A-Initial Dye Concentration	854.07	1	854.07	8.55	0.0105	
B-pH	1825.79	1	1825.79	18.29	0.0007	
C-Temperature	618.85	1	618.85	6.20	0.0250	
D-Urea	639.74	1	639.74	6.41	0.0230	
AB	1575.89	1	1575.89	15.78	0.0012	
AC	21.55	1	21.55	0.22	0.6489	
AD	261.23	1	261.23	2.62	0.1266	
BC	885.21	1	885.21	8.87	0.0094	
BD	127.07	1	127.07	0.27	0.2770	
CD	207.00	1	207.00	2.07	0.1704	
A^2	17214.50	1	17214.50	172.43	<0.0001	
B^2	14055.56	1	14055.56	140.79	<0.0001	
C^2	14055.56	1	17043.15	170.71	<0.0001	
D^2	17043.15	1	11.94	0.12	0.7343	
Residual	11.94	15	99.83	-	-	

Lack of Fit	1497.52	10	149.57	414.95	<0.0001	Significant
Pure Error	1495.72	5	0.36	-	-	
Cor Total	1.80	29	-	-	-	
	47711.15					

Std. Dev.	9.99	R-Squared	0.9686
Mean	40.75	Adj R-Squared	0.9393
C.V. %	24.52	Pred R-Squared	0.8194
PRESS	8617.96	Adeq Precision	16.960

Figure 6.15: ANOVA results of Methyl Orange (MO) de-colorization efficiency.



Design Summary

Study Type	Response Surface	Runs	30
Initial Design	Central Composite	Blocks	No Blocks
Design Model	Quadratic		

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	Initial Dye conce	mg/l	Numeric	10.00	100.00	-1.000	1.000	55.000	40.249
B	pH	units	Numeric	4.00	9.00	-1.000	1.000	6.500	2.236
C	Temperature	oC	Numeric	25.00	50.00	-1.000	1.000	37.500	11.180
D	Urea	g% w/v	Numeric	0.00	3.00	-1.000	1.000	1.500	1.342

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Dye decolorizati	%	30	Polynomial	1.02	99.87	40.7507	39.8795	97.9118	None	Quadratic

Figure 6.16: Design summary of Central Composite Design (CCD).

Factor	*Name*	*Level*	*Low Levels*	*High Level*	*Std. Dev.*	*Coding*	
A	*Initial Dye Concentration*	*10.00*	*10.00*	*100.00*	*0.000*	*Actual*	
B	*pH*	*9.00*	*9.00*	*9.00*	*0.000*	*Actual*	
C	*Temperature*	*50.00*	*25.00*	*50.00*	*0.000*	*Actual*	
D	*Urea*	*3.00*	*0.00*	*3.00*	*0.000*	*Actual*	
Response	*Prediction*	*SE Mean*	*95%CI Low*	*95% CI high*	*SE Pred*	*95%PI Low*	*95%PI high*
Dye decolorization	*57.1288*	*9.06*	*37.83*	*76.43*	*14.92*	*25.33*	*88.93*

Figure 6.17: Point Prediction Table

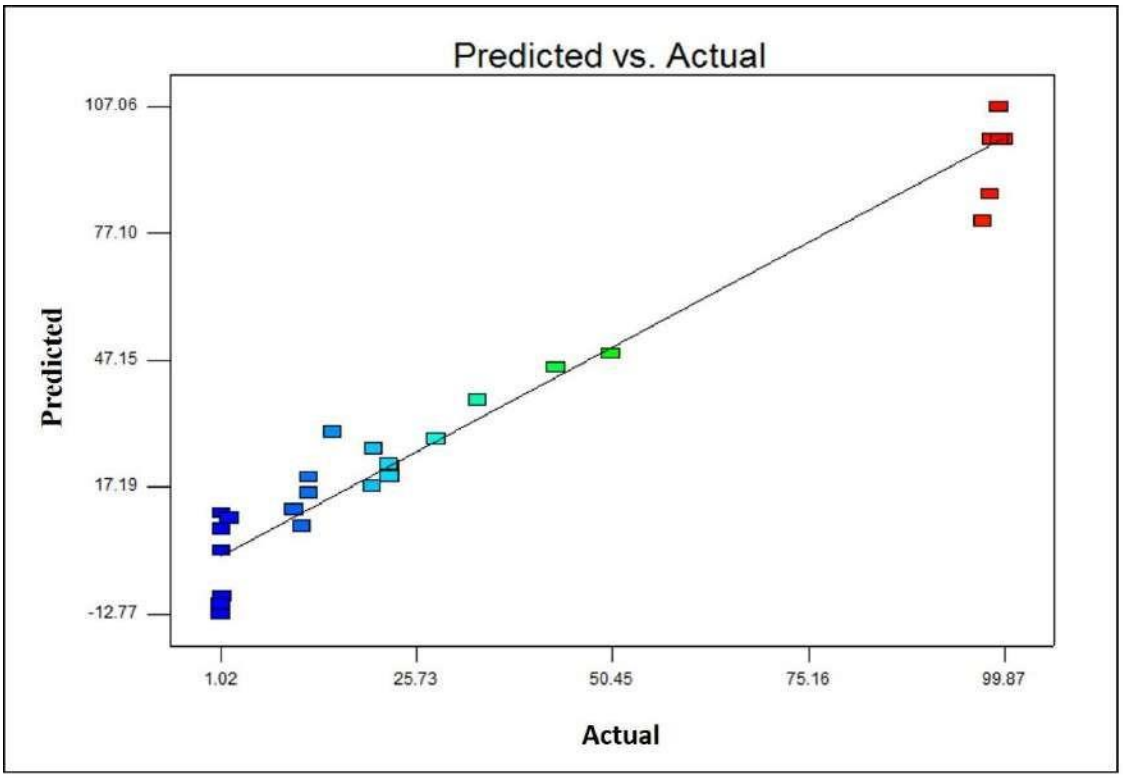


Figure 6.18: Predicted vs actual plot

Table 6.4: Response Surface Methodology results showing optimized conditions for dye removal. Run 1: Computational data predicted by RSM design, Run 2: Experimental data

Run	Initial dye concentration (mg/l)	pH	Temperature (°C)	Extra nitrogen source (Urea) g% w/v	Dye removal%
1	55	6.50	37	1.5	88.93
2	55	6.50	37	1.5	93.05

In this study, among eleven bacterial strains, *Bacillus cereus* J4 demonstrated the capability to remove up to 89.8% of methyl orange from aqueous solutions. Our observations revealed that several physical and chemical parameters significantly influence the rate of methyl orange de-colorization. According to statistical analysis, key variables for efficient de-colorization include the initial dye concentration, temperature, and additional nitrogen sources

(urea), and pH. Given these findings, *Bacillus cereus* J4 shows promise as an efficient agent for the bioremediation of industrial effluents containing MO, potentially contributing to safer environmental discharge practices.

Objective 3: Assessment of the possible mechanism involved in the dye removal process.

6.6 Assessment of mechanisms involved in the dye removal process

Fourier transform infrared spectroscopy (FTIR), (FESEM), and (HPLC) were utilized to study the components and characteristics of the samples (Hashemi et al., 2022).

6.6.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analyses was carried out on Methyl Orange dyes at a scan speed of 16 to verify the presence of various functional groups in the mid-IR range of 400–4000 cm^{-1}. Spectroscopically pure KBr pellets were used to prepare the samples. The FTIR spectra were used to identify the various functional groups present in both the original MO dye and its metabolites. A 20 ml aliquot was taken from each flask after full decolorization, centrifuged, and the supernatant was oven-dried for a whole night at 50°C (Kalyani et al., 2008). The dried sample was then mixed with KBr in a ratio of 5:95 (w/w), ground, and fused into pellets prepared under vacuum conditions using a PCI hydraulic press. FTIR spectrum of the untreated MO dye sample exhibited several characteristic absorption peaks: At 2920 cm^{-1}, the absorption indicates C-H stretching vibrations from alkanes. The 1646 cm^{-1} peak is characteristic of C=C stretching in alkenes or C=O stretching in amides. The wavenumber at 1600 cm^{-1} is associated with C=C stretching in aromatic rings, while 1516 cm^{-1} corresponds to N-O asymmetric stretching in nitro compounds or additional C=C stretching in aromatics. The 1361 cm^{-1} peak indicates C-H bending vibrations in methyl groups or symmetric N-O stretching in nitro compounds. The absorption at 1184 cm^{-1} can be indicative of C-O stretching in esters, ethers, and alcohols, whereas 1031 cm^{-1} is characteristic of C-O stretching in similar functional groups. The 817 cm^{-1} and 690 cm^{-1} peaks correspond to C-H bending vibrations in aromatic rings, specifically out-of-plane bending in mono-substituted and para-substituted rings, respectively (Cyril et al., 2019).

The FTIR spectra of post-treatment of the methyl orange dye exhibited distinct peaks: The absorption at 3313 cm^{-1} is indicative of O-H stretching vibrations. The 2102 cm^{-1} peak is characteristic of C≡C stretching. The wavenumber at 1642 cm^{-1} is associated with C=C stretching vibrations. The absorption at 585 cm^{-1} is usually linked to C-Br stretching. In comparison to the control group, the study revealed that some peaks shifted, some completely disappeared, and few additional peaks appeared, as illustrated in Figure 6.20. This indicates that the isolated bacterium *Bacillus cereus* J4 is effectively degrading the Methyl Orange dye (Figure 6.20). A recent research study demonstrated similar degradation pathways of azo dyes using bacterial strains, where changes in the FTIR spectra indicated the breakdown of dye molecules (Ali et al., 2020). Another study showed that the disappearance and transformation of specific FTIR peaks are indicative of effective microbial degradation of complex dye structures (Zhang et. al., 2018). These FTIR data highlight Bacillus cereus J4's potential for bioremediation applications, especially for the breakdown of hazardous dyes like methyl orange (MO), which helps to improve industrial effluent management procedures.

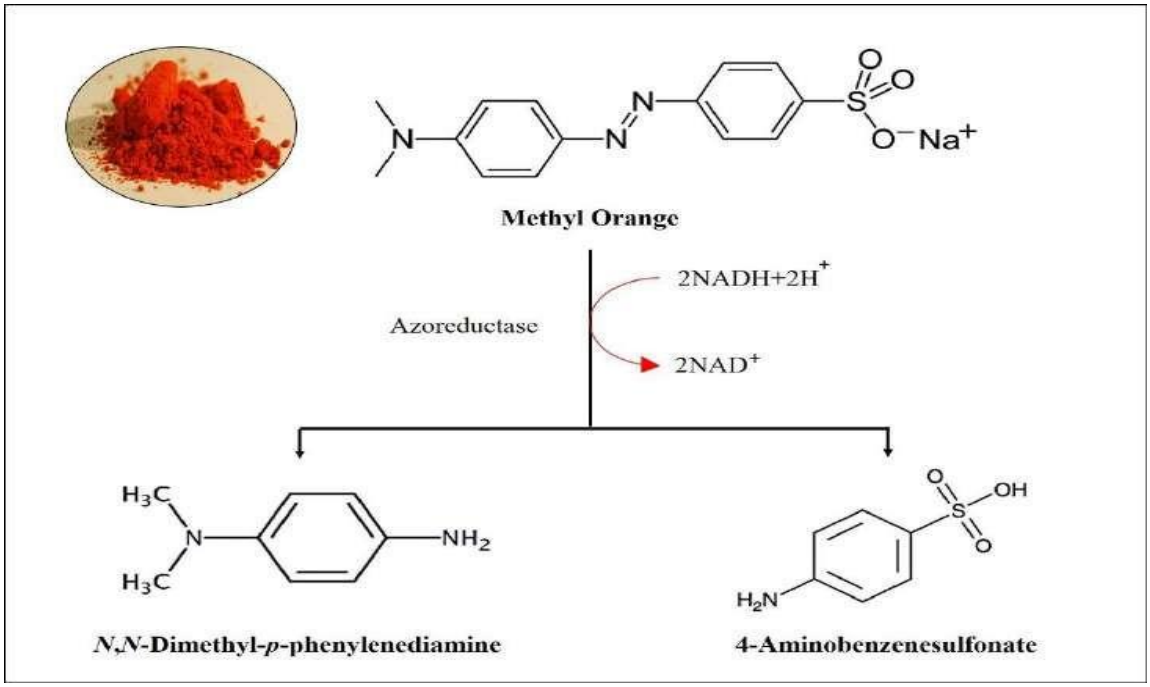


Figure 6.19: Reductive cleavage of methyl orange

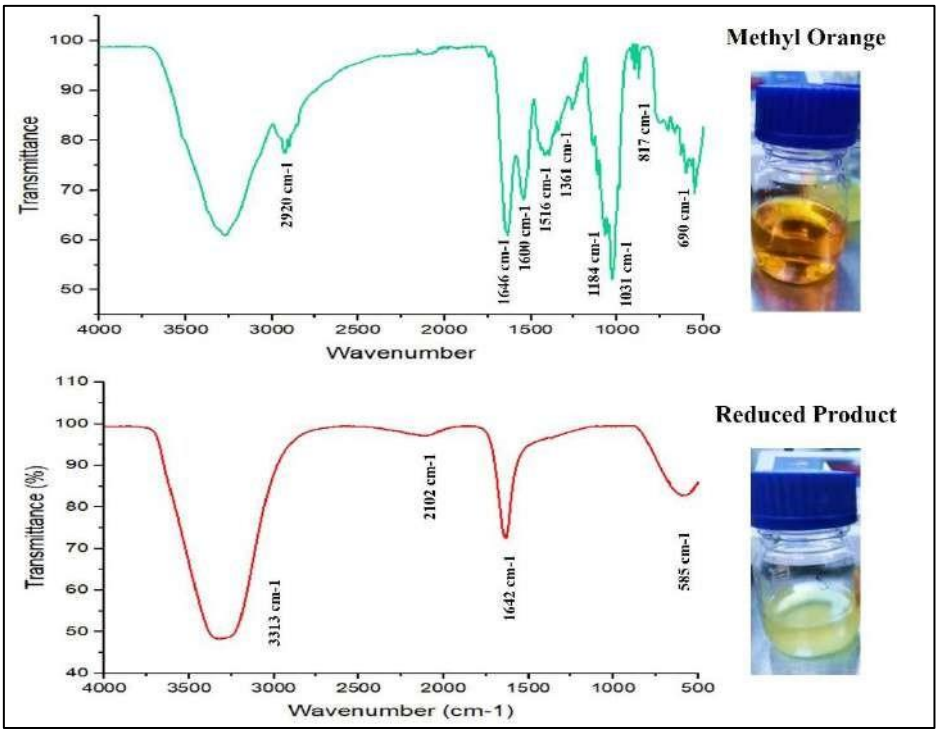


Figure 6.20: FTIR Spectrum of untreated and treated Methyl orange.

6.6.2 High-performance liquid chromatography

High-Performance Liquid Chromatography (HPLC) analyses was conducted using a C18 column (symmetry, 4.6 mm × 250 mm) with a gradient elution of methanol and acetonitrile (75:25) at a flow rate of 1 ml/min for 10 minutes. Detection was performed using UV detectors set at 254 nm (Song et al., 2003). This approach was employed to analyze the decolorized dye products and confirm the extent of Methyl Orange degradation and the presence of intermediate products. The HPLC chromatogram of pure Methyl Orange dye displayed multiple distinct peaks at retention times of 5.611 and 6.048 minutes. These peaks correspond to the different components and structural configurations of the Methyl Orange molecule (Figure 6.21). After the decolorization process, significant changes were found in the analysis of the treated dye solution. Specifically, a new peak emerged at a retention time of 9.504 and 10.176 minutes, indicating the formation of new degradation products. The appearance of this new peak, along with the disappearance of

reduction of the original peaks suggests the complete breakdown (biodegradation) of the azo dye MO.

The chromatogram of decolorized dye metabolites showing multiple peaks indicates the formation of various intermediate products during the biodegradation process. This observation aligns with previous research studies that have investigated the biodegradation of azo dyes. For instance, a study by Zhang et al. (2019) reported similar findings where the biodegradation of azo dyes by bacterial strains resulted in the formation of intermediate compounds, as evidenced by the appearance of new peaks in HPLC chromatograms. The efficacy of biodegradation was further confirmed by the appearance of distinct peaks corresponding to the degradation metabolites. In a different study, HPLC analysis showed that when azo dyes were broken down by microbes, intermediate metabolites were formed (Ali et al., 2020). The chromatographic analysis performed in this study demonstrates that *Bacillus cereus* J4 effectively degrades Methyl Orange, leading to the formation of various intermediate products. These results underscore the potential of *Bacillus cereus* J4 as a bioremediation agent for the treatment of industrial effluents containing MO dye. The findings are consistent with recent research studies that highlight the importance of using microbial strains for the biodegradation of toxic dyes in wastewater treatment applications.

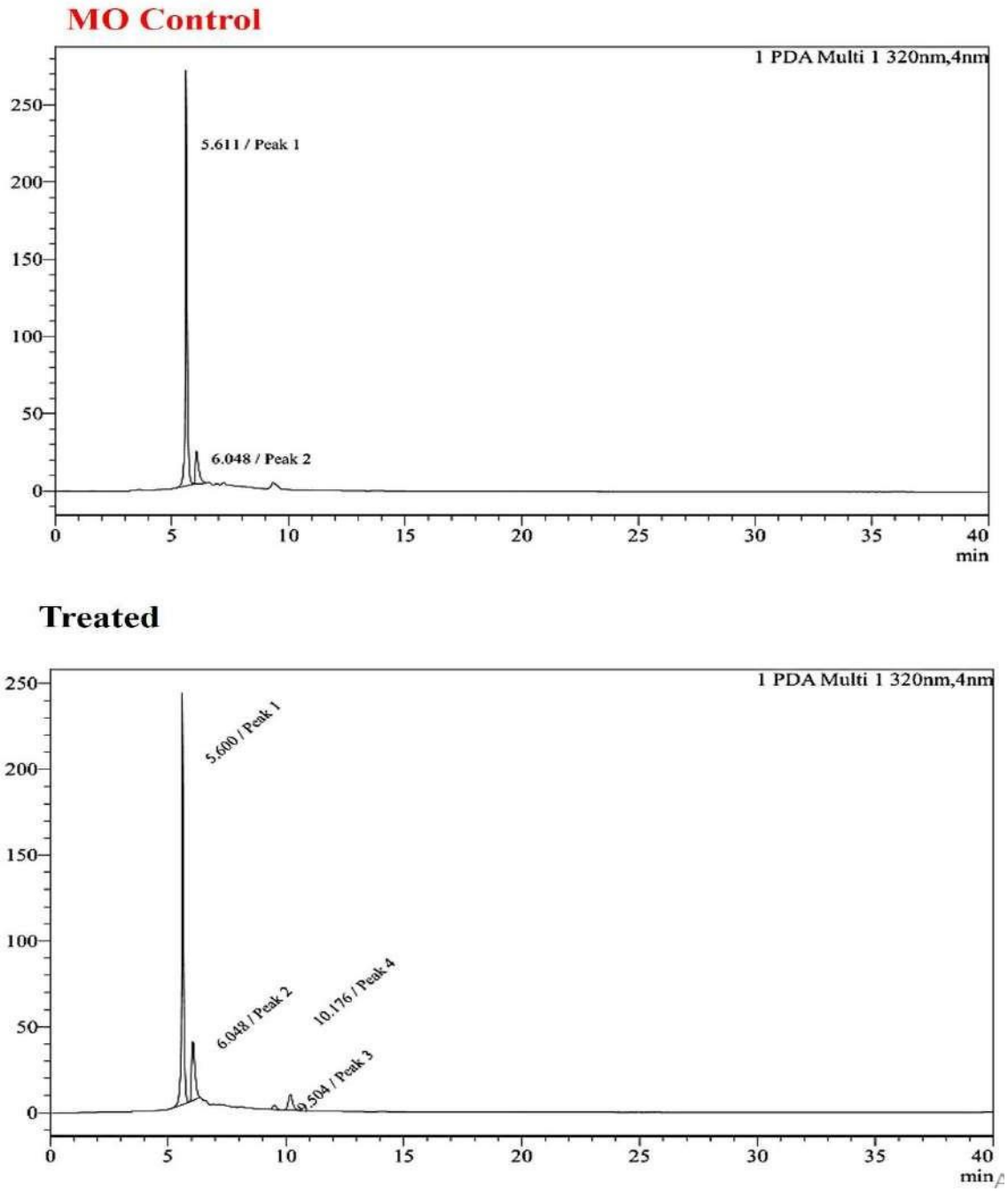


Figure 6.21: HPLC chromatogram of pure methyl orange and treated solution.

6.6.3 Changes in surface characteristics revealed by FE-SEM

The morphological changes in the surface of live *Bacillus cereus* J4 cells before and after dye treatment were analyzed using Field emission scanning electron microscopy (FE-SEM). The FE-SEM images provided insights into the structural alterations that occurred due to the interaction with Methyl Orange dye. In the control images, the cell wall of *Bacillus cereus* J4 appeared uniform and smooth, characteristic of gram-positive bacteria. The peptidoglycan layer, which ranges between 20 and 80 nm in thickness, was visible and consistent across the surface, indicating a healthy and intact bacterial cell structure. Post-treatment images revealed significant morphological changes in the bacterial cells. The cell wall became thick, uneven, and rough compared to the smooth surface observed in the control cells. Methyl Orange dye molecules adhering to the bacterial cell wall cause deformation and roughening of the surfaces of the cells. The dye molecules seemed to be densely packed on the surface, indicating strong interactions between the functional groups on the cell surface (such as -COO, -NO, or -NH2 groups) and the dye molecules. These structural changes indicate that the presence of the dye induces considerable stress on the bacterial cells, altering their surface morphology. The distortion and unevenness observed in the treated cells imply that the dye molecules may disrupt the cell wall integrity, potentially interfering with normal cellular functions. This is consistent with findings from other studies where the interaction of azo dyes with bacterial cells led to noticeable changes in cell morphology (Kalyani et al., 2008; Lade et al., 2012). The thickening of the cell wall observed in the FE-SEM images suggests that *Bacillus cereus* J4 cells might be attempting to fortify their cell walls in response to the toxic stress imposed by the Methyl Orange dye. This response could be a defensive mechanism to prevent further damage or to compartmentalize the dye molecules away from vital cellular processes. Recent research by Zhang et al. (2019) showed similar morphological alterations in bacterial cells exposed to azo dyes, where the cell surface became irregular and thickened due to dye adsorption. Another study demonstrated that the interaction between bacterial cell wall components and dye molecules could lead to significant surface morphological changes, impacting the overall cell viability and function (Ali et al., 2020). FE-SEM analysis of *Bacillus cereus* J4 before and after Methyl Orange dye treatment highlights significant morphological

changes indicative of cellular stress and dye adsorption. These findings, supported by recent literature, underscore the impact of azo dye interactions on bacterial cell structure and emphasize the importance of understanding these mechanisms for effective bioremediation strategies.

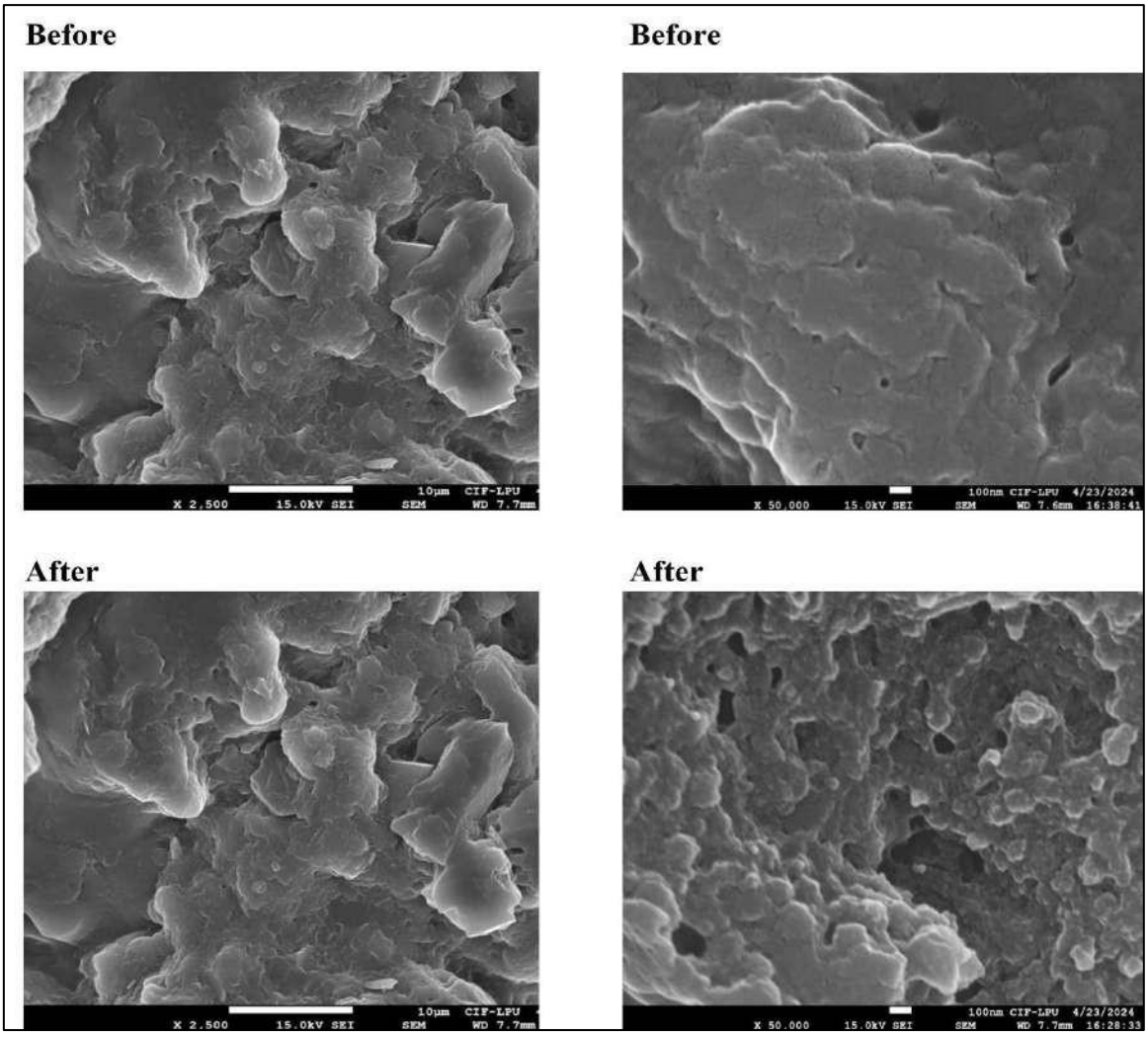


Figure 6.22: FE-SEM images of the surface of live *Bacillus cereus* J4 cells before and after dye treatment

Objective 4: Toxicity analysis of the treated solution on selected agriculturally important crop seed germination.

6.7 Phytotoxicity studies of native and treated Methyl Orange (MO) solution

Phytotoxicity studies of native and treated Methyl Orange (MO) solutions were conducted to evaluate their effects on plant germination and growth. The study involved various plant species, including *Cicer arietinum* (Chickpea), *Vigna radiata* (Mung bean), *Sorghum bicolor* (Jowar), *Trigonella foenum-graecum* (Methi), and *Vigna unguiculata* (Red Lobia), among others. The results highlighted the significant differences in germination rates and root lengths between plants exposed to treated and untreated MO solutions.

In the treated sets, using distilled water, 100% germination was observed in *Cicer arietinum, Vigna radiata, and Sorghum bicolor, and 90% germination in Trigonella foenum-graecum* (Methi) and *Vigna unguiculata*. The average root lengths for these plants were 16.12 ±
0.23 mm and 32 ± 0.10 mm, respectively. These results indicate that the treatment of MO dye significantly reduces its toxicity, making it less harmful to plant growth. The treated MO solution exhibited a germination rate of 80% in *Vigna unguiculata* (Black-eyed Pea) and 60% in *Phaseolus aconotifolius* (Moth Bean), with root lengths of 8 ± 0.24 mm and 43 ± 0.23 mm, respectively. Although there was a reduction in germination compared to the distilled water treatment, the treated MO solution still supported a considerable degree of plant growth, indicating a partial mitigation of the dye's phytotoxic effects. In contrast, the native MO solution exhibited high toxicity, severely impacting germination and root development in several plant species. *Vigna radiata* (Mung bean), *Sorghum bicolor* (Jowar), *Vigna unguiculata* (Black-eyed Lobia), *Brassica juncea* (Indian mustard), *Hordeum vulgare* (Barley), *Vigna unguiculata* (Red Lobia), *Zea Mays* (Corn), and *Phaseolus aconotifolius* (Moth Bean) experienced germination rates as low as 0%, 20%, and 15%, respectively (Figure 6.23). The adverse effects were also evident in the stunted root lengths (Table 6.5).

Based on the study of phytotoxicity, it can be concluded that certain plants, such as *Brassica juncea*, *Vigna radiata*, and *Sorghum bicolor*, are more sensitive to native MO dye, showing severely reduced germination rates and root lengths. However, the treatment of MO dye with *Bacillus cereus* J4 significantly improved its toxicity, resulting in higher germination rates and healthier root growth in these sensitive plant species. This suggests

that the bioremediation of MO dye using *Bacillus cereus* J4 cells is effective in reducing its phytotoxicity, making the treated dye less harmful to agricultural and environmental health. Reportedly, the bioremediation of azo dyes, including Methyl Orange, using bacterial strains resulted in reduced phytotoxicity and improved plant growth. Similarly, another study demonstrated the effectiveness of bacterial degradation in mitigating the toxic effects of industrial dyes on plant species (Gupta and Nayak, 2021). These studies reinforce the potential of *Bacillus cereus* J4 in the safe release of treated industrial effluents into the environment, promoting sustainable agricultural practices. Overall, the phytotoxicity studies clearly illustrate the detrimental effects of native MO dye on plant germination and growth and highlight the significant reduction in toxicity achieved through bacterial treatment, emphasizing the potential of *Bacillus cereus* J4 in environmental bioremediation applications.

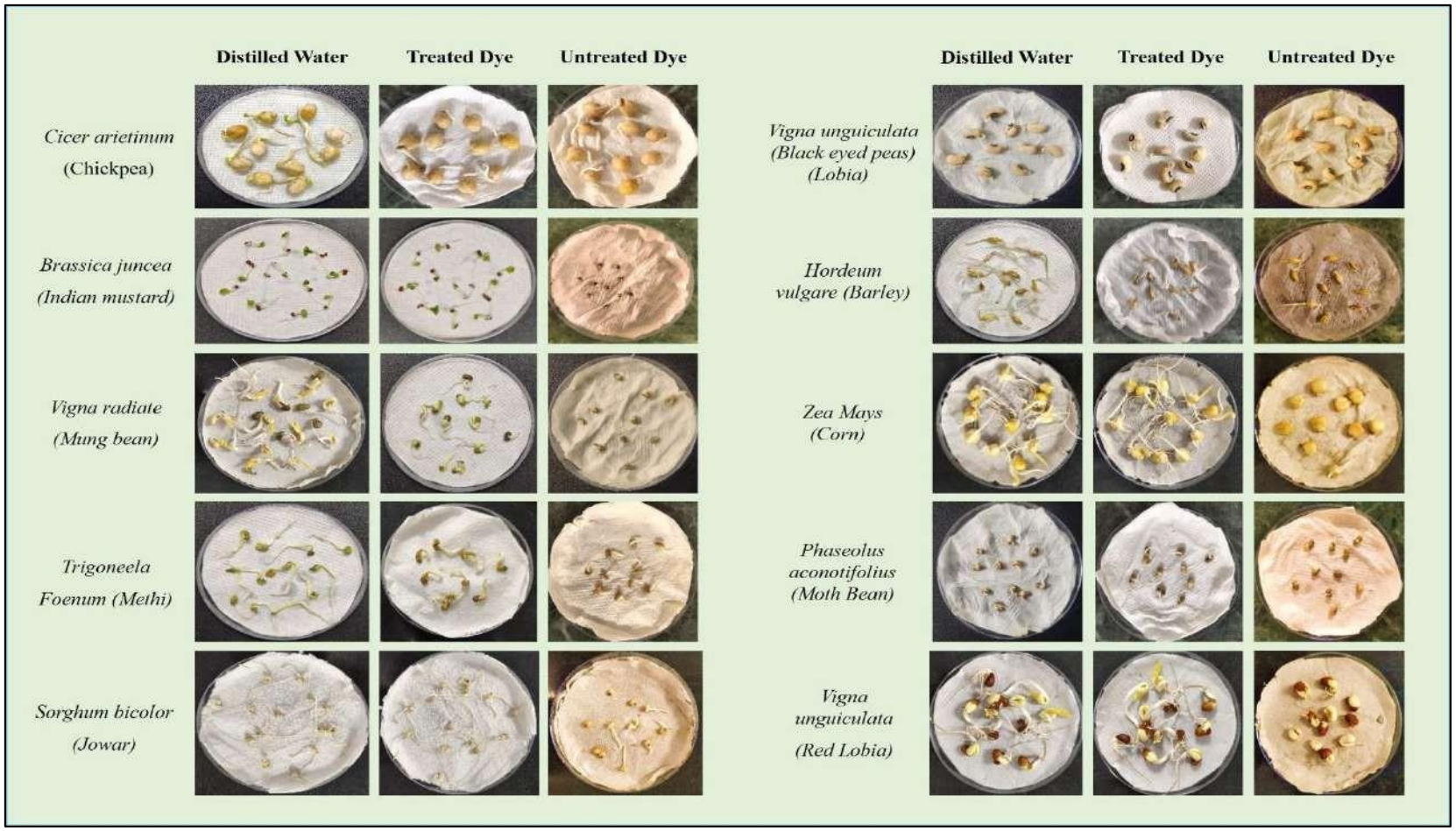


Figure 6.23: Phytotoxicity study in plant seeds (*Cicer arietinum* (Chickpea), *Vigna radiata* (Mung bean), *Trigonella Foenum (*Methi), *Sorghum bicolor* (Jowar), *Brassica juncea* (Indian mustard), *Vigna unguiculata* (Black-eyed peas), *Hordeum vulgare* (Barley)*, Zea Mays* (Corn), *Phaseolus aconotifolius (*Moth Bean), and *Vigna unguiculata* (Red Lobia).

Table 6.5: Measurements of phytotoxicity analysis

Plants	Treatment Type	Germination (%age)	Radical (mm)	Plumule (mm)
Cicer arietinum **(Chickpea)**	Water	100%	52 ± 0.22	3 ± 0.18
	Untreated MO solution	20%	7 ± 0.56	4 ± 0.24
	Treated MO solution	100%	24 ± 0.81	3 ± 0.15
Brassica juncea **(Indian mustard)**	Water	100%		34 ± 0.3
	Untreated MO solution	0		
	Treated MO solution	100%	20 ± 0.07	2 ± 0.02
Vigna radiata **(Mung bean)**	Water	100%	45 ± 0.84	7 ± 0.33
	Untreated MO solution	0		
	Treated MO solution	100%	21 ± 0.81	3 ± 0.15
Trigoneela Foenum (**Methi)**	Water	100%	33.58 ± 1.67	6.47 ± 0.13
	Untreated MO solution	20%	7 ± 0.56	8 ± 0.12
	Treated MO solution	90%	16.12 ± 0.23	4.12 ± 0.42
Sorghum bicolor **(Jowar)**	Water	100%	26 ± 0.21	5 ± 0.14
	Untreated MO solution	0%		
	Treated MO solution	100%	19 ± 0.31	0.25
Vigna unguiculata (Black eyed) **(Lobia)**	Water	100%	10 ± 0.23	3 ± 0.12
	Untreated MO solution	0%		
	Treated MO solution	60%	8 ± 0.24	4 ± 0.31
Hordeum vulgare (Barley)	Water	100%	34 ± 0.24	10 ± 0.05
	Untreated MO solution	0%		
	Treated MO solution	100%	27 ± 0.32	5 ± 0.11
Phaseolus aconotifolius	Water	100%	57 ± 0.16	8 ± 0.32

(Moth Bean)	Untreated MO solution	15%	8 ± 0.21	6 ± 0.13
	Treated MO solution	80%	43 ± 0.23	9 ± 0.05
Vigna unguiculata **(Red Lobia)**	Water	100%	52 ± 0.11	13 ± 0.21
	Untreated MO solution	0%		
	Treated MO solution	90%	32 ± 0.10	9 ± 0.01
Zea Mays **(Corn)**	Water	100%	41 ± 0.11	13 ± 0.21
	Untreated MO solution	0%		
	Treated MO solution	90%	22 ± 0.10	6 ± 0.01

6.8 Microbial Toxicity

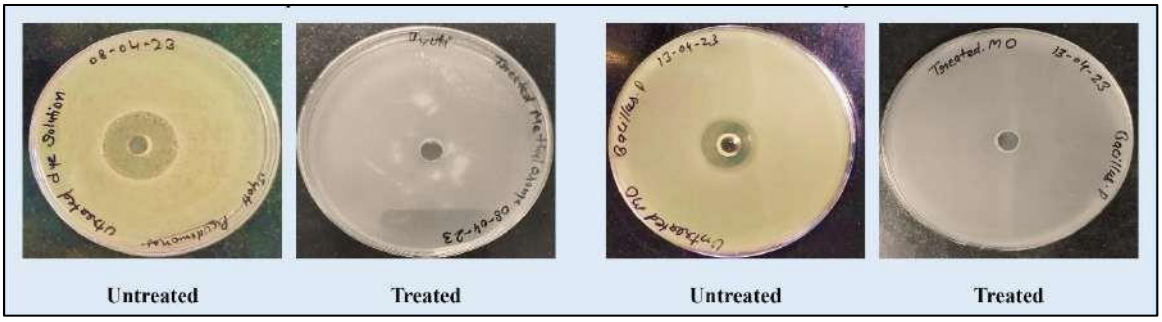
The microbial toxicity test was conducted to assess the toxicity of treated and untreated Methyl Orange (MO) dye solutions against two microbial strains: *Pseudomonas* sp. MTCC2421 and *Bacillus* sp. MTCC441. These strains were chosen due to their prevalence and beneficial roles in the rhizosphere, which is crucial for plant health. The results, as shown in Table 6.6 and Figure 6.24, indicated no zone of inhibition when the MO dye solution was treated with *Bacillus cereus* J4 cells. In contrast, untreated MO dye exhibited significant toxicity, with inhibition zones of 25 ± 2 mm and 15 ± 2 mm for *Pseudomonas* sp. and *Bacillus* sp., respectively. This stark difference in the inhibition zones suggests that the treatment of MO dye with *Bacillus cereus* J4 substantially reduces its toxicity. The reduction in microbial toxicity can be attributed to the biodegradation process facilitated by *Bacillus cereus* J4. During this process, the bacterium breaks down the MO dye into less toxic metabolites, as evidenced by the disappearance and transformation of specific absorption peaks in FTIR spectra. Bacterial enzymes likely interact with dye (MO) molecules during the detoxification process, breaking down toxic functional groups and creating safe byproducts. The absence of inhibition zones for treated MO dye indicates that the detoxified products are less harmful to beneficial rhizosphere microorganisms. *Pseudomonas sp.* and *Bacillus sp.* are essential to the rhizosphere's nutrient cycling, plant growth promotion, and disease control. Therefore, the reduced toxicity of treated MO dye ensures the preservation of these microbial functions, indirectly promoting plant health and growth.

Recent study demonstrated that bacterial degradation of azo dyes, including MO, resulted in non-toxic byproducts that did not inhibit the growth of beneficial soil microbes (Sharma et al., 2021). Similarly, another study reported that bio-remediated dye solutions exhibited minimal microbial toxicity, thereby supporting soil microbial health and plant productivity (Raj et al., 2022). The reduction in toxicity benefits the rhizosphere region by maintaining the viability and function of beneficial microorganisms, *Pseudomonas* sp. and *Bacillus* sp. Consequently, the treated MO dye is less likely to harm the soil ecosystem, thereby indirectly supporting plant health. This study underscores the potential of *Bacillus*

cereus J4 in bioremediation applications aimed at mitigating the environmental impact of industrial dye pollutants.

Table 6.5: Measurements of phytotoxicity analysis

	Pseudomonas sp. MTCC2421	*Bacillus sp. MTCC441*
Inhibition zone of untreated MO dye (mm)	25 ± 2	15 ± 2
Inhibition zone of treated MO dye (mm)	No Zone	No Zone
Cork borer diameter (mm)	6 ± 1	6 ± 1



Pseudomonas sp. MTCC2421

Bacillus sp. MTCC441

Figure 6.24: MO untreated and treated MO plates of *Pseudomonas sp. MTCC2421*; MO untreated and treated MO plates of *Bacillus sp. MTCC441*

Objective 5: Immobilization of the isolated strain for possible remediation of textile industry effluent.

6.10 Batch De-colorization Study

Maximizing dye removal efficiency is crucial for various industries to mitigate environmental pollution. Our study focused on the use of immobilized culture beads to enhance the removal rates of Methyl Orange (MO) dye. Conducted under sterile conditions, each treatment was tested three times to ensure accuracy. The culture beads, prepared by immobilizing *Bacillus cereus* J4, were cleaned three times with sterile water to eliminate contaminants. An MO solution with an initial dye amount (concentration) of 50 mg/l was treated over ten days at 35°C using different numbers of these beads (20, 30, 40, 50, 60, and 70) (Figure 6.25), (Figure 6.26). The outcomes showed a clear relationship between the number of immobilized culture beads and the dye removal efficiency. Using 20 beads, the bacterial strain achieved a minimum dye removal rate of 40%. With 30, 40, and 50 beads, the dye removal rates increased to 54%, 74%, and 79%, respectively. Remarkably, the use of 60 beads resulted in more than 80% dye removal. This improvement is attributed to the greater surface area and higher bacterial biomass provided by the increased number of beads, which facilitates more efficient dye degradation. Supporting our findings, past studies have demonstrated similar results wherein Liu et al. (2021) found that immobilizing bacterial cells in alginate beads enhanced the biodegradation of textile dyes. Additionally, another study reported that increasing the number of immobilized microbial beads improved the removal efficiency of various pollutants, including azo dyes like MO (Zhang et al., 2022). Increasing the number of immobilized culture beads significantly enhanced the removal rate of Methyl Orange dye. This method offers a practical and scalable solution for industries to achieve higher dye removal efficiency, thereby contributing to more sustainable practices and reducing the environmental impact of dye pollutants.

Figure 6.25: (a) Na-alginate beads of culture bacteria (J4), (b) after treatment, and (c) Control dye.

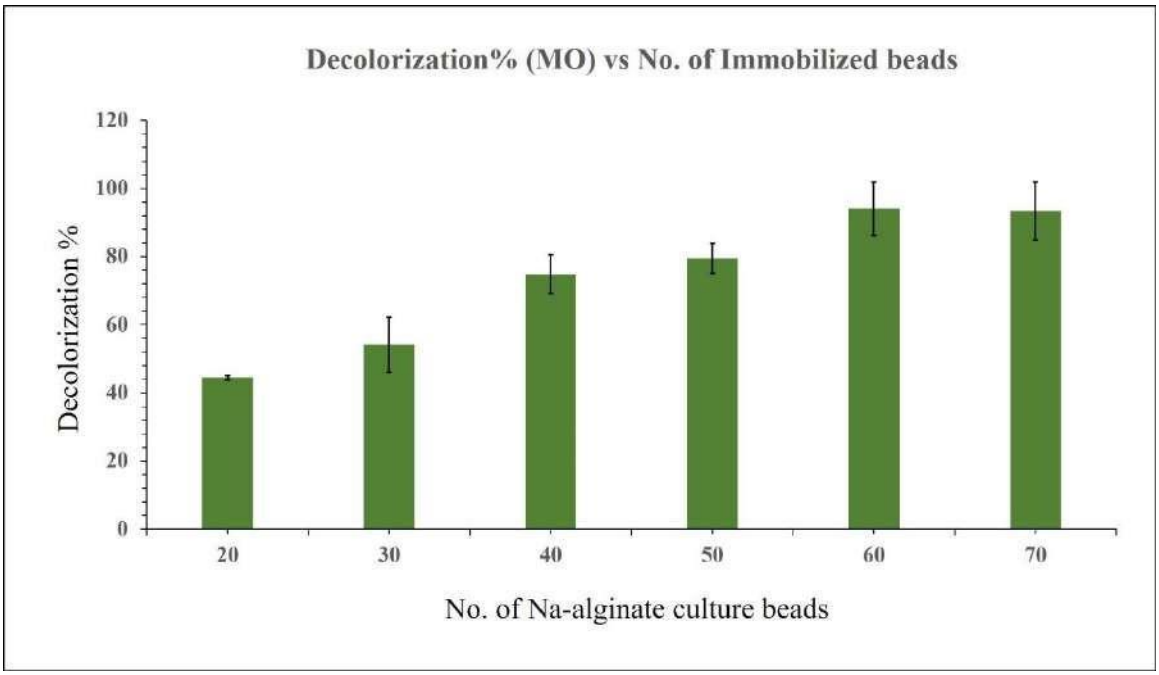


Figure 6.26: De-colorization percentage of methyl orange with respect to the number of Immobilized beads (Batch study)

6.11 Column studies with Methyl Orange

Column studies were carried out to assess the adsorption capacity of sodium alginate (Na-Alg) immobilized culture beads for the continuous removal of MO dye from textile effluent. The experimental setup involved a glass column with dimensions of 50 cm in length and 1.3 cm in diameter, filled with the immobilized culture beads. The MO solution, with an optimized dye concentration of 50 ppm and a pH value of 7, flowed through the column at controlled flow rates of 0.5 ml/minute and 1ml/minute using a peristaltic pump (Garg et al., 2024).

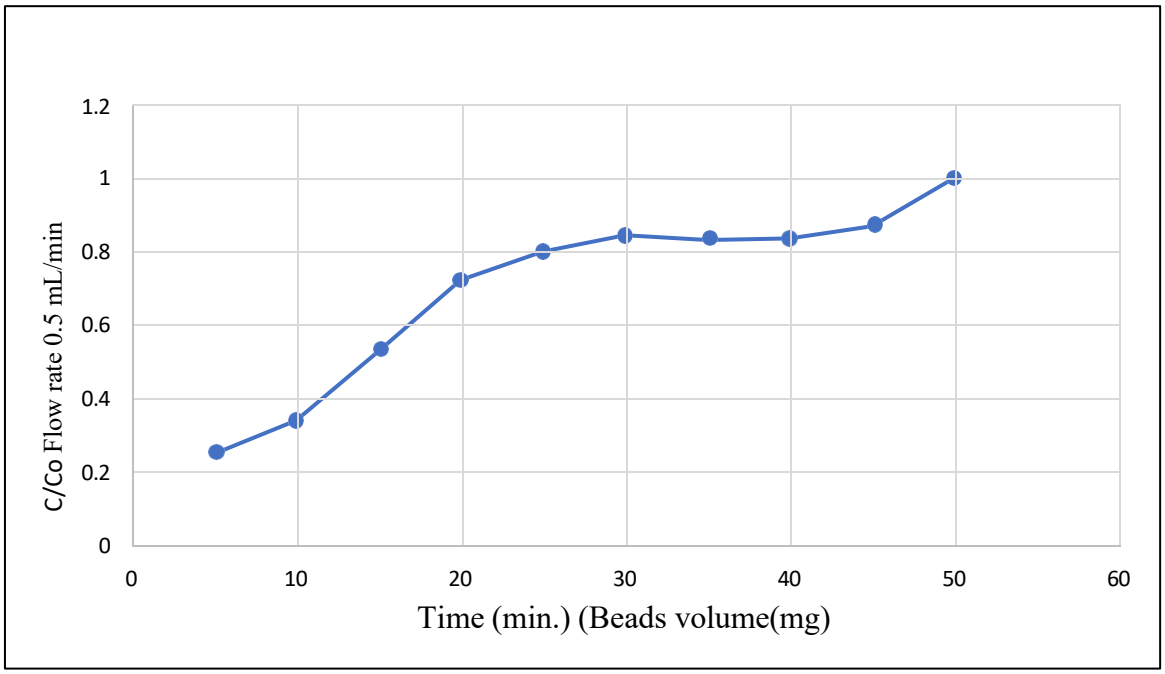


Figure 6.27: Breakthrough curve Time vs C/Co (Beads volume and Flow rate 0.5 ml/min)

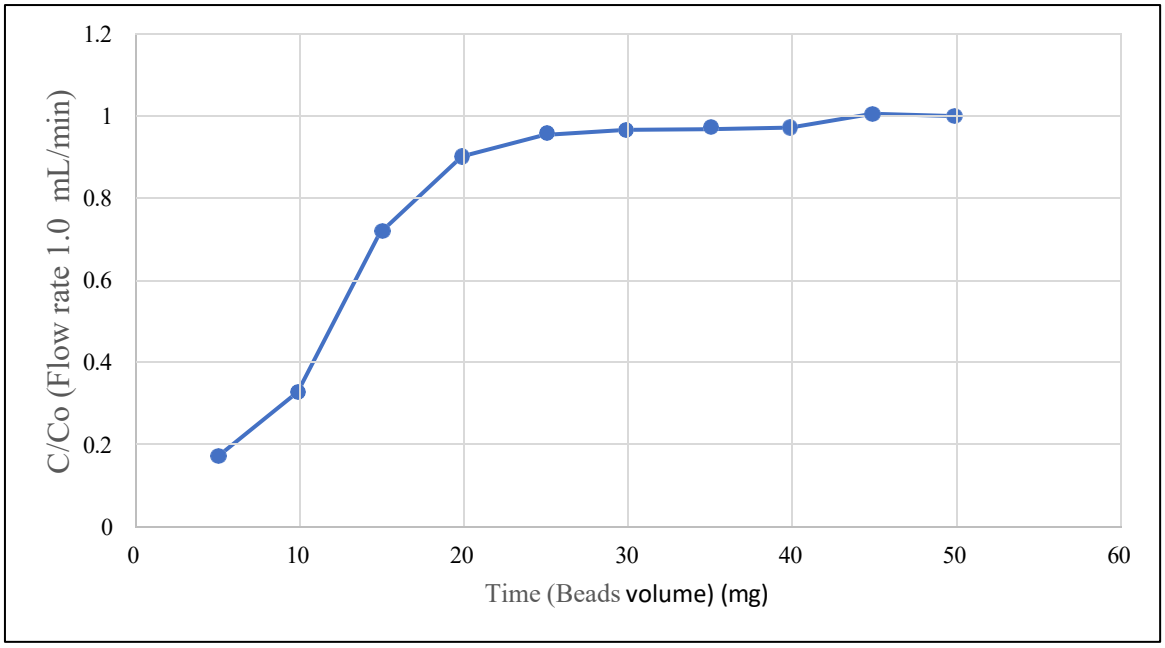


Figure 6.28: Breakthrough curve Time vs C/Co (Beads volume and Flow rate (1.0 mL/min)

The results demonstrated an equilibrium adsorption capacity of 44.26 mg/g and a dye removal percentage of 75.01%. In comparison, batch system experiments using 60 immobilized culture beads showed a significantly higher dye removal efficiency of 94.04% for the same initial dye concentrations of 50 ppm. The discrepancy between the column and batch systems can be attributed to the salts in the MO dye solutions. These salts likely reduced the adsorption efficiency by competing with MO dye molecules for adsorption sites on the Na-Alginate immobilized beads.

Recent studies support these findings, indicating that continuous flow systems often exhibit lower adsorption capacities than batch systems due to the dynamic interactions in a flowing medium. A research study highlighted that the presence of competing ions in continuous flow conditions can significantly impact the adsorption efficiency of pollutants (Liu et al., 2022). Likewise, additional research found that the adsorption performance of immobilized beads in column studies was hindered by the ionic strength of the solution, which aligns with our observations (Zhang et al., 2021). Overall, column studies provide valuable

insights into the practical application of adsorption systems under continuous flow conditions; however, the presence of salts in the effluent can markedly reduce the adsorption efficiency of MO dye. This underscores the importance of optimizing operational parameters and potentially pre-treating effluents to remove interfering substances for achieving higher dye removal efficiencies in industrial applications.

6.12 FESEM characterization of Immobilized beads before and after treatment:

The results obtained from scanning electron microscopy reveal changes in the surface topology of sodium alginate immobilized blank beads (those without bacteria) before and after the dye adsorption treatment. The FESEM images of the blank beads displayed an amorphous nature and heterogeneous morphology. In contrast, the morphology of the immobilized bacterial beads (containing bacteria) altered significantly, as illustrated in Figure 6.29 (a). In the magnified portion of the circled area, small rod-shaped bacteria can be observed within the cross-section of the beads. It was found that bacterial immobilization led to the swelling of the biomaterial, which facilitated the penetration of bacteria into the micropores. The bacterial cells were immobilized within alginate beads through a natural phenomenon known as adhesion. Furthermore, the scanning electron micrograph of the bacterial co-immobilized sample demonstrated that immobilization was not homogeneously distributed throughout the material structure. Instead, it was preferentially located in specific regions characterized by rough and porous structures. The rough and porous characteristics of the raw material enabled microorganisms to attach more securely than they could to smoother surfaces. This behavior has also been documented in previous studies involving single cultures (Genisheva et al., 2011). Moreover, earlier research has indicated that immobilization technology is beneficial for sustaining the degradation efficiency of strains in wastewater treatment (Xu et al., 2012). After conducting adsorption experiments with immobilized culture beads (containing bacteria) and a dye solution, the SEM figure 6.29 (a and b). revealed a crumbling of the beads' surface morphology. This finding suggests that the bacteria were capable of biodegrading and disintegrating the dye over a specific period of time.

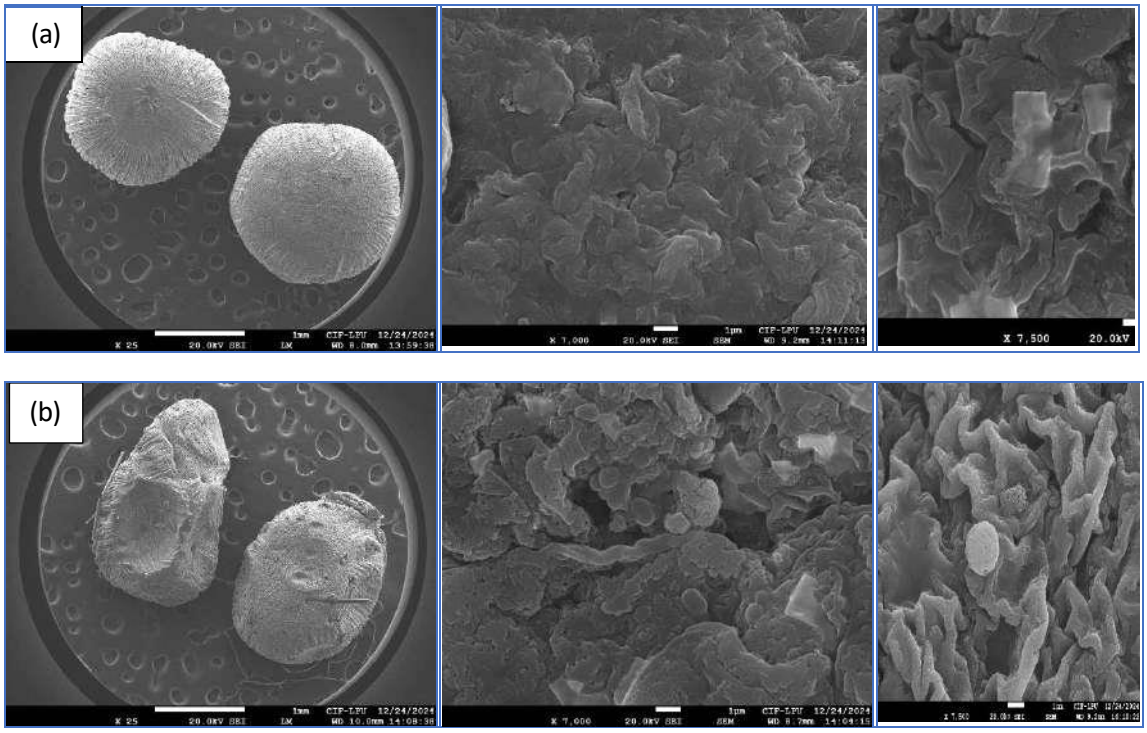


Figure 6.29: FESEM images of Immobilized culture beads; (a) Immobilized beads before dye treatment, (b) Immobilized beads after dye treatment

CHAPTER 7

CONCLUSION &FUTURE REMARKS

Conclusion & Future Remarks

This study aims to explore the potential use of a novel bacterial strain for the biodegradation and elimination of methyl orange, a common and persistent azo dye often found in wastewater effluents (Sudarshan et al., 2023; Pande et al., 2019). Samples of industrial effluent taken from the industrial area of Panipat City revealed the presence of eleven different bacterial strains. Among these strains, *Bacillus cereus* J4 OQ392442 was singled out for its bioremediation potential. The ability of ***Bacillus cereus* J4 OQ392442** to significantly reduce methyl orange (MO) was investigated in the liquid phase. The study examined various physicochemical parameters for dye removal, including initial dye concentration (5 − 100 mg/l), Inoculum percentage (0.5-2.5 v/v), Incubation time (8-48hrs), pH (4-9), temperature (25 −45°C), and agitation speed (0 - 200 rpm). Furthermore, chemical factors such as extra nitrogen source (peptone, sodium nitrate, ammonium nitrate, ammonium sulfate, and urea) and extra carbon source (D-Glucose, D-fructose, Maltose, mannitol, and Sucrose) were taken into consideration at concentrations ranging from 0 % to 3% and were optimized through PB and RSM. According to the study, at an initial pH of 7.0, temperature of 37°C, Initial concentrations of 50 mg/l, and an extra nitrogen source of 2% (w/v), 89.05% MO removal was attained. FT-IR and FE-SEM studies revealed potential removal and surface groups (viz. −NH and- NO) in bacterial chemical sorptive behavior. For extensive removal prospects, a continuous removal bed column study was conducted using batch and whole-cell immobilized alginate beads as packing material for the column, achieving a 50.15% MO removal at two flow rates of 1ml/minute and 0.5 ml/minute. Additionally, toxicity in plants like (*Cicer arietinum, Brassica juncea, Vigna radiata, Trigonella foenum-graecum, Sorghum bicolor, Vigna unguiculata, Hordeum vulgare, Phaseolus aconitifolius, Vigna unguiculata, and Zea mays)* and microbial toxicity (*Bacillus subtilis and Pseudomonas fluorescens)* studies revealed that MO treated with Bacillus cereus J4 was significantly less toxic than its native form in terms of environmental and agricultural aspects. Overall, the study indicates that *Bacillus cereus* J4 could make a significant contribution to textile industries and may have a key role in environmental bioremediation in the future.

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Appendix

Sodium alginate beads

A density of 109 cfucm3 was achieved by dispersing healthy Bacillus cereus J4 cells in a sterile Na alginate solution. Under constant stirring, the suspension was extruded through a succession of needles with a 0.2 mm diameter and a 0.5 cm3min−1 rate in a sterile 0.1M CaCl2 solution. After being left in the solution for ten minutes, the beads were racked and cleaned with sterile distilled water. Subsequently, these beads were then used as packing for the column that was being used to continue the process of removing the Methyl Orange dye.

Nutrient Agar

Nutrient Agar is used as a general-purpose medium for the cultivation of less fastidious microorganisms and can be enriched with blood or other biological fluids.

Composition

Ingredients	**Grams / L**
Peptone	5.000
Sodium chloride	5.000
peptone B#	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

- Equivalent to Beef extract Directions: Suspend 28.0 grams in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45.2-50°C.

Nutrient Broth (NB)

A liquid medium called nutritional broth is used to develop a diverse variety of organisms from clinical samples and others materials. For certain uses, this medium can be enhanced with additional substances like blood, carbohydrates, etc. It is also employed for sterility testing.

Composition

Ingredients	**Grams / Liter**
Peptones	10.000
Beef extract	10.000
Sodium chloride	5.000
Final pH	7.3 ± 0.1

For 1000 milliliters of filtered or distilled water, suspend 25 gm. If needed, apply heat to completely melt the medium. You can autoclave to sterilize it for 30 minutes at 10 pounds of pressure (115°C) or 15 minutes at 15 pounds of pressure (121°C).

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Response surface methodology for rapid removal of an azo dye methyl orange by indigenous bacterial strain (*Bacillus cereus* J4)

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ABSTRACT

Sulfonated azo dyes like Methyl Orange (MO) are widely used in industries such as textiles, paper, food, and printing, but their discharge into wastewater poses a serious threat to aquatic life. This study aimed to isolate and optimize bacterial strains for the bioremediation of MO from textile effluents. Eleven bacterial strains were isolated from industrial effluents in Panipat, India, with *Bacillus cereus* J4 demonstrating the highest decolorization efficiency, removing up to 98% of MO within 48 hours. To optimize the decolorization process, two methods were employed Plackett–Burman (PB) design and Response Surface Methodology (RSM). PB design identified significant factors affecting decolorization, while RSM provided a more comprehensive optimization by considering interactions among factors. RSM reported an optimal removal rate of 89.8% MO at 50 mg/l dye concentration, pH 7, 37°C, and 2% urea. In conclusion, *Bacillus cereus* J4 showed promising potential for MO bioremediation under optimized conditions. Future studies should explore scaling up the process and examining the strain's effectiveness on other industrial dyes to develop a broad-spectrum bioremediation strategy.

1. INTRODUCTION

Methyl orange (MO) is a sulfonated azo dye extensively utilized in textile dyeing facilities and as a pH indicator in biochemical applications [1]. Despite its utility, MO and its metabolites pose

due to their cost-effectiveness, non-toxic byproducts, and potential for complete mineralization of dyes [12–15].

Microbial dye removal generally involves either the degradation of dyes by microbial cells or the adsorption of dyes onto microbial

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