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BIODEGRADATION OF TEXTILE AZO DYES USING BACTERIAL STRAINS ISOLATED FROM TEXTILE EFFLUENT AND IMPROVEMENT OF SEEDLING GROWTH

Antony P. J. and Joshua Kariyil Jolly*

Department of Zoology, St. Michael's College, Alappuzha, India.

***Corresponding Author: Joshua Kariyil Jolly**
Department of Zoology, St. Michael's College, Alappuzha, India.

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ABSTRACT
The exponential expansion of industrial sectors and subsequent advancements have resulted in a substantial upsurge in the release of chemical waste into the natural surroundings. The release of wastewater from industrial activities is a significant factor in the contamination of water and soil, hence engendering an adverse ecological setting that detrimentally impacts the plant and animal life inside ecosystems. The discharge of textile wastewater from textile manufacturers is a significant environmental concern among many industrial emissions. In light of the aforementioned environmental difficulties, biotreatment techniques have emerged as a well recognised and efficacious approach for the disposal of undesirable colour and toxicity found in textile effluents. In contrast to traditional treatment procedures, biotreatment technologies provide enhanced cost-effectiveness and environmental friendliness, making them a more desirable choice for the purpose of sustainable waste management. The present investigation focuses on the degradation process of two frequently used textile dyes, namely Methyl Red (MR) and Malachite Green (MG), via the utilisation of bacteria that have been isolated from textile effluents. This research identified and isolated six different bacterial strains from the effluent samples and subjected them to dye decolorization assays. Remarkably, the isolated bacteria, identified as Pseudomonas spp., demonstrated the capability to decolorize both Methyl Red (MR) and Malachite Green (MG) dyes. This finding suggests that Pseudomonas spp. could be promising candidates for bio-treatment applications to remediate dye-contaminated wastewater from the textile industry.

KEYWORD: Azo dye; Degradation; Methyl Red; Malachite Green; Textile effluent, Absorbance.

INTRODUCTION
Environmental pollution is widely recognised as a significant issue with profound socio-economic and health implications. Water pollution is a significant environmental issue that warrants considerable attention. The observation of colour is widely recognised as a prominent and easily discernible indicator of water pollution. The potential harm caused by the release of effluents containing synthetic dyes with intense coloration has been extensively studied.[1] Azo dyes find extensive applications across various industries such as food, pharmaceutical, cosmetic, textile, and leather. Synthetic compounds are defined by the presence of one or more intramolecular NQN bonds.[7] In the dyeing process, a significant proportion of dyes, ranging from 5% to 10%, are discharged into wastewater streams associated with textile production. These wastewater streams eventually make their way into natural water bodies.[2] Azo dyes are known for their extensive colour range, enabling the production of vibrant and eye-catching clothing. These dyes possess exceptional colorfastness, allowing garments to maintain their hues over an extended period. This remarkable stability is attributed to their high resistance to light exposure and repeated washing, ensuring that the colours remain vivid and intact. Moreover, it is worth noting that the vibrant hue exhibited by discharge dyes, even when present in minute concentrations, exerts a significant influence on the surrounding aquatic ecosystem. The release of these products has been found to have a long-lasting effect on the ecological balance of the system due to the resulting reduction in light penetration and photosynthesis.[3] The agricultural sector has been significantly impacted by the discharge of textile effluents from a growing number of textile industries. The impact of textile effluent on plants exhibits a range of outcomes, including reduced crop yield and the wilting of young plants.

In the realm of textile wastewater treatment, a multitude of physical and chemical methods have been explored to effectively eliminate dyes. These methods include the utilisation of Fenton's reagent, ozonation, photochemical reactions, sodium hydrochlorite, silica gel, ion exchange, and several others. The methods employed for effluent treatment are associated with several drawbacks. These include the generation of sludge, the formation of by-products, limited effectiveness for certain types of dyes, the necessity of high concentrations of dissolved oxygen, and restricted applicability to a specific range of scenarios.[4] The recognition and exploitation of microorganisms' ability to degrade and metabolise a diverse range of substances have been extensively studied and utilised in various biotreatment processes. The cost effectiveness and diverse metabolic pathways, as well as the versatility of microorganisms involved, make microbiological treatment methods highly appealing.[5] The method described in this study demonstrates its utility in achieving the degradation of various dyes in an environmentally friendly manner. The investigation into crop responses to industrial effluent holds potential practical significance in the field of agriculture.

MATERIALS AND METHODS

Collection of soil samples

The unprocessed textile waste was gathered from little dyeing operations. The industry releases dye- and toxin-filled effluents with a dark blue-black hue into the open air. In less than an hour, the samples were collected in sterile plastic cans and sent to the lab. The acquired sample was preserved in order to isolate and test several bacteria with the capacity to degrade dyes.

Chemicals and media

The textile dyes employed in this experimental study include Malachite Green (MG) and Methyl Red (MR). One commonly employed medium for studying culture is known as Nutrient agar media. This medium consists of a combination of various components, including 0.5g of Yeast, 1g of Tryptone, 1g of NaCl, and 2g of Agar powder. The chemicals and dyes utilised in this study were procured from HI media laboratories, located in Mumbai, India.

Isolation of dye degrading bacterial isolates

In this study, a volume of 1 ml from each dye effluent sample was extracted and transferred into separate conical flasks. To create serial dilutions, 10 ml of distilled water was added to each flask. The dilutions ranged from 10-1 to 10-6. In this study, a meticulous selection process was employed to obtain serially diluted samples with concentrations of 10−3, 10−4, and 10−5. These samples were then transferred onto sterile petri plates using the spread plate method, ensuring a controlled and standardised approach for the experimental setup.

Screening using Spread Plate Method

In this experiment, a sterile unsolidified nutrient agar was poured into a petri dish. The agar was then allowed to solidify and the dish was placed in a laminar air flow environment. In accordance with established laboratory protocols, a volume of 0.1 ml was extracted from the dilution series 10^-3. This aliquot was carefully transferred using a pipette onto the central region of the agar plate's surface. In this experimental procedure, an L-shaped glass spreader was immersed into a solution of alcohol and subsequently subjected to a controlled flame using a Bunsen burner. In order to ensure an even distribution of samples, the sterile glass spreader was utilised to distribute the samples evenly across the plates. Simultaneously, the petri dish was rotated underneath to facilitate the distribution process. The experimental procedure was replicated for dilutions of 10^-4 and 10^-5. The experiment involved incubating three distinct agar plates, each with unique properties, along with a control plate. The incubation process was carried out at a temperature of 37°C for a duration of 24 hours. Following the incubation period, the bacterial colonies were observed and identified.

Isolating bacteria found in dye effluent

There were six types of morphologically different bacterial colonies found in the culture.

Screening of bacterial isolates using Streak Plate

In this study, a total of six petri dishes were meticulously prepared to ensure optimal conditions for microbial growth. The petri dishes were carefully filled with nutrient agar media, a commonly used medium that provides essential nutrients for microbial proliferation. To maintain a sterile environment, the petri dishes were placed inside a laminar air flow chamber, which effectively minimises the risk of contamination by providing a continuous flow of filtered air. This controlled setup guarantees the integrity of the experiment and allows for accurate observations and analysis of microbial growth. In this study, bacteria were selected from six separate colonies, each exhibiting unique morphological features as observed in the preceding stage. To ensure accuracy and precision, a loop was utilised to individually transfer the bacteria onto the pre-prepared petri dishes. The bacteria were distributed evenly on each plate using a streaking technique. In this experiment, the plate was rotated by 90 degrees, and the loop was carefully passed through the streaked area two to three times. The loop was subsequently employed to generate a distinctive "zig-zag" arrangement within the remaining portion of the plate, while ensuring no contact with the previously streaked area. The aforementioned procedure was iteratively executed until the entirety of the plate's surface was saturated with the distinctive zig-zag configuration. Following the experimental procedure, the six agar plates were subjected to incubation at a temperature of 37°C for a period of 24 hours. Following the incubation period, bacterial colonies exhibiting robust growth were selected and subjected to isolation and purification procedures on individual plates. These purified bacterial cultures were then stored at 4°C for future use.

Dye Decolorization experiments

In this experiment, two sets of six test tubes were prepared, with each test tube containing 6 ml of nutrient broth. These test tubes were subjected to autoclaving, a process that involves exposing them to high temperature and pressure. Specifically, the autoclaving was performed at a temperature of 121°C and a pressure of 15 lbs for a duration of 15 minutes. The test tubes, which had been autoclaved, were carefully introduced into the laminar air flow environment. In the experimental setup, the initial batch of test tubes was subjected to treatment with Methyl Red dye, while the subsequent batch received treatment with Malachite Green dye. In this study, a series of test tubes containing Methyl Red (MR) were assigned the labels Jo1, Jo2, Jo3, Jo4, Jo5, and Jo6. Similarly, the test tubes containing Malachite Green (MG) were designated as G1, G2, G3, G4, G5, and G6. These labels were used to differentiate and track the various samples throughout the experiment. The test tubes were subsequently inoculated with bacterial inoculum from each individual isolate. In this study, the test tubes were placed in a mechanical shaker and incubated at a temperature of 37 ºC. The pH of the environment was maintained at a level of 7. The duration of the incubation period was set at 2 days. In this study, samples were collected at regular intervals of 24 hours for the purpose of observation. In this study, a volume of approximately 5 ml of the dye solution was subjected to a filtration process followed by centrifugation at a speed of 4000 revolutions per minute (rpm) for a duration of 10 minutes. The assessment of decolourization was conducted by utilising a spectrophotometer to measure the absorbance at specific wavelengths for the dyes methyl red (MR) and malachite green (MG). The absorbance at 480 nm was measured for MR, while the absorbance at 613 nm was measured for MG. These wavelengths correspond to the maximum absorbance (λm) for each respective dye. The measurements were performed on the supernatant obtained from the decolourization process.

De-Colourization Assay

The quantification of decolorization in the experiment was conducted utilising a spectrophotometer, a commonly employed instrument in scientific research for measuring the absorption of light by a substance. The obtained results were expressed as a percentage, indicating the proportion of the initial dye concentration that was eradicated or removed by the bacterial strains under investigation. The experimental protocol involved the measurement of the absorbance of the dye solution before and after exposure to the bacterial strains with dye-degrading capabilities. The initial absorbance value was determined based on the concentration of the dye in the solution at the beginning of the experiment. After a suitable incubation period, the final absorbance measurement was recorded following the exposure to the bacterial strains.[6]

The percentage decolourization was calculated from the following formula,

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

The observation of a higher percentage of decolorization suggests that the bacterial strains exhibited a more effective degradation of the dye. The investigation focused on evaluating the decolorization percentage and its implications for the effectiveness of the dye degradation process. Additionally, it aimed to assess the proficiency of the selected bacterial strains in converting dyes into less harmful chemicals or colourless substances. The obtained results revealed significant findings in these areas. The decolorization test results were instrumental in understanding the potential of the identified bacterial strains for bioremediation and their effectiveness in reducing dye pollution in industrial wastewater.

Identification of selected bacterial strains

The process of identifying bacterial strains capable of degrading dyes entailed a thorough methodology that incorporated several aspects, including morphological analysis, Gramme staining, and assessment of biochemical properties. Every stage of the identification method yielded significant data for differentiating and classifying the bacterial strains.

Growth promoting test

The investigation advanced to assess the growth-promoting characteristics of the chosen dye-degrading bacterial strains in black gramme seeds. In order to perform the assessment, the isolates were introduced into a nutrient broth medium to facilitate their proliferation and expansion. Following this, the bacterial cultures were inoculated onto the test petri plates, which were supplemented with black gramme seeds and Methyl Red (MR) and Malachite Green (MG) dyes, respectively. To facilitate comparison, two control plates devoid of bacterial presence were constructed for each colour, beside two test plates that were inoculated with bacteria. The petri plates were thereafter placed in an incubator for a duration of five days, during which the seeds were given the opportunity to undergo germination and proliferation in the presence of the corresponding colours and bacterial cultures. Throughout the experiment, many growth indicators were precisely measured for both the control and dye-infused petri plates. These measurements included root length, shoot length, weight, and leaf count. The growth parameters that were recorded were afterwards subjected to analysis and compared between the control plates and the test plates that were treated with dye and bacterial inoculation. The objective of this investigation was to investigate the impact of bacterial strains capable of degrading colours on the germination and growth of black gramme seeds in the presence of MR and MG dyes. The objective of this research was to examine and compare the growth results of different bacterial strains in order to understand their potential in enhancing the growth of black gramme seeds and mitigating the negative impacts of dye contamination on plant development.

RESULTS AND DISCUSSION

Isolation of microorganisms with dye degrading potential

In order to identify new compounds with the potential to degrade dye in wastewater, bacterial colonies were obtained using the serial dilution approach and then screened using the spread plate method. The screening procedure identified the existence of six unique bacterial species with varying physical characteristics inside the culture. Each of these bacterial isolates exhibited unique characteristics in terms of colony shape, size, color, and texture. The discovery of these diverse microorganisms suggests their natural adaptation and survival in the presence of toxic dyes, indicating their potential for dye degradation. To ensure the purity of the isolated bacterial colonies and facilitate further studies, each isolated colony was sub-cultured on agar medium using the streak plate method and preserved in a refrigerator at 4°C.

Determination of decolorization efficiency

Those isolates namely 1, 2, 3, 4, 5 and 6 were further assayed for decolorization efficiency. Decolorization efficiency of all 6 isolates were studied and the results are visualized in.

Fig 01. Methyl Red after 24 hours.

Fig 02. Methyl Red after 48 hours.

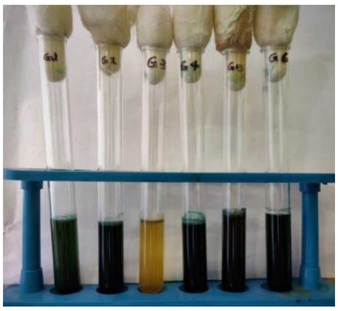

Fig 03. Malachite Green after 24 hours.

Fig 04. Malachite Green after 48 hours.

De-Colourization Assay

The initial absorbance reading is denoted as C, while the final absorbance readings are as follows: Jo1, Jo2, Jo3, Jo4, Jo5, Jo6, G1, G2, G3, G4, G5, G6; where Jo series represent MR treated test tubes whereas G series represent MG treated test tubes.

The Spectrophotometer measures the absorbance of Methyl Red (MR) at 480nm in the presence of over 80 units of distilled water.

Table 1: Methyl Red 48 hours.

Test tube name	Absorbance
C	0.621
Jo1	0.521
Jo2	0.251
Jo3	0.137
Jo4	0.476
Jo5	0.433
Jo6	0.554

Test tube names and their respective absorbance readings are presented in Table 1, representing MR Dye's behavior over 48 hours (about 2 days). As bacteria decolorize the dye, the color intensity transitions from light to dark, with Jo2 being the lightest and Jo6 being the darkest. The order is as follows: Jo3 → Jo2 → Jo5 → Jo4 → Jo1 → Jo6.

The Spectrophotometer measures the absorbance of Malachite Green (MG) at 613nm in the presence of over 80 units of distilled water.

Table 2: MG Dye in 48 hours.

Test tube name	Absorbance
C	1.832
G1	1.116
G2	1.424
G3	0.379
G4	1.688
G5	1.683

G6	1.755

Test tube names and their respective absorbance readings are presented in Table 2, representing the MG Dye's behavior over 48 hours (about 2 days). As bacteria decolorize the dye, the color intensity transitions from light to dark, with G3 being the lightest and G6 being the darkest. The order is as follows G3 → G1 → G2 → G5 → G4 → G6.

MR Dye Decolourization percentage

Table 3: MR Dye 48 hours.

Test tube name	% Decolourization
Jo1	16.103%
Jo2	59.521%
Jo3	77.938%
Jo4	23.349%
Jo5	30.273%
Jo6	10.789%

MG Dye Decolourization percentage

Table 4: MG Dye 48 hours.

Test tube name	% Decolourization
G1	39.089%
G2	39.082%
G3	79.312%
G4	7.86%
G5	8.133%
G6	4.203%

As a result, certain isolates were found to have the capability to degrade both dyes, Methyl Red (MR) and Malachite Green (MG), to some degree within a 48-hour timeframe. Among these isolates, third bacterial strains demonstrated the highest level of dye decolorization, accomplishing it in a significantly shorter time compared to the other selected isolates under similar conditions.

Identification of bacterial isolate

The third bacteria isolated from the bacterial culture exhibits the most effective dye-degrading properties with both Malachite Green (MG) and Methyl Red (MR). The identification of this specific bacterium was carried out using morphological and biochemical properties, following the standard protocol outlined in Bergey's Manual.[8]

Biochemical characteristics

To identify the bacterial isolates, Gram staining and biochemical tests were conducted. The integration of Gramme staining and biochemical assays yielded significant data that facilitated the precise identification of the bacterial isolates. Table 5 presents the results of the biochemical testing.

Table 5: Biochemical Characterization of Screened Isolated Bacteria.

Test	Result
Gram Staining	Negative (–ve)
Indole	Negative (–ve)
MR	Negative (–ve)
VP	Negative (–ve)
Catalase	Positive (+ve)
Citrate utilization	Positive (+ve)

The bacterial isolate, which exhibited a morphology characterised by a short rod form, underwent a comprehensive analysis of its characteristics, comparing them to the descriptions provided in Bergey's Manual. According to the findings of this investigation, the bacterial strains have been effectively classified as members of the Pseudomonas spp. taxonomic group.

Seedling Growth Results

The growth-enhancing characteristics of black gramme seeds were assessed after a five-day treatment with Pseudomonas spp. in dye effluent. Two experimental configurations were used for the purpose of comparison: the Control plate, consisting only of dye and black gramme seeds, and the Test plate, including black gramme seeds, dye, and Pseudomonas spp.

Following a five-day treatment period, the growth parameters of the black gramme seeds were assessed and then compared between the Control and Test plates. It was anticipated that the Test plate would include Pseudomonas spp., which may facilitate enhanced development and perhaps manifest advantageous impacts on the seeds when exposed to dye effluent. The findings of this assessment would provide valuable insights into the possible use of Pseudomonas spp. for promoting the development of black gramme seeds in settings polluted with dyes.

Fig 5. CONTROL Methyl Red.

Fig 6. TEST Methyl Red.

Fig 7. CONTROL Malachite Green.

Fig 8. TEST Malachite Green.

The growth characteristics of the black gramme seeds were evaluated after a period of 5 days. The elements that were taken into consideration for assessment encompassed.

1. Shoot length: It refers to the measurement of the aerial section of the plant, extending from the base to the apex of the shoot.
2. Root length: It refers to the measurement of the subterranean segment of a plant's root system, extending from the base to the apex of the longest root.
3. Weight (g): The weight of the black gramme seedlings was measured in grammes, representing the biomass generated over the course of five days.
4. Leaf Count: The quantification of leaves seen on individual black gramme seedlings, serving as an indicator of the plant's growth and leaf generation.

The growth parameters were measured and afterwards compared between the Control plate and the Test plate.

Table 6: CONTROL Methyl Red.

Sample	Shoot length (cm)	Root length (cm)	Weight (g)	No of Leaves
1	16.2cm	2.8cm	0.10g	2
2	18cm	5.0cm	0.13g	2
3	23.8cm	8.6cm	0.23g	2
4	19cm	4.6cm	0.21g	2
5	14.4cm	3.7cm	0.15g	2
6	12.2cm	4.2cm	0.13g	2

Table 7: TEST Methyl Red.

Sample	Shoot length (cm)	Root length (cm)	Weight (g)	No of Leaves
1	4.4cm	2.0cm	0.09g	2
2	9.3cm	3.7cm	0.12g	2
3	4.1cm	2.8cm	0.11g	2
4	6.2cm	3.7cm	0.06g	2
5	6.0cm	1.1cm	0.13g	2
6	5.3cm	2.3cm	0.10g	0

Table 8: CONTROL Malachite Green.

Sample	Shoot length (cm)	Root length (cm)	Weight (g)	No of Leaves
1	15.2cm	6.3cm	0.15g	2
2	17.9cm	4.6cm	0.13g	2
3	18.4cm	3.6cm	0.14g	2
4	6.0cm	3.2cm	0.09g	2
5	5.7cm	4.3cm	0.16g	0
6	5.2cm	3.5cm	0.12g	2

Table 9: TEST Malachite Green.

Sample	Shoot length (cm)	Root length (cm)	Weight (g)	No of Leaves
1	17.8cm	6.8cm	0.19g	2
2	14.7cm	3.5cm	0.16g	2
3	15.6cm	7.1cm	0.22g	2
4	7.0cm	2.2cm	0.10g	2
5	8.6cm	1.6cm	0.09g	2
6	7.2cm	2.4cm	0.12g	1

The findings derived from the assessment of the seed characteristics provide significant insights into the impact of Pseudomonas spp. on the development of black gramme plants in the presence of dye. The treatment of Pseudomonas spp. had varying effects on the two dyes, Methyl Red (MR) and Malachite Green (MG).

The introduction of Pseudomonas spp. in the context of MR resulted in an adverse impact, manifesting as inhibited growth in the seedlings of black gramme. This observation implies that the presence of Pseudomonas spp. in the MR dye effluent may impede or suppress the development of the seedlings, resulting in diminished seedling quality.

In contrast, the use of MG dye demonstrated advantageous outcomes upon the incorporation of Pseudomonas spp. The seedlings of black gramme that were subjected to treatment with Pseudomonas spp. exhibited improved seedling quality along with a marginal growth enhancement. This observation suggests that Pseudomonas spp. may exhibit growth-enhancing or protective properties on the seedlings when exposed to MG dye.

In general, the findings indicate that the impact of Pseudomonas spp. on black gramme seedlings is contingent upon the specific dye (MR or MG) found in the effluent. Additional research is necessary in order to get a comprehensive understanding of the underlying processes that give rise to these divergent outcomes, as well as to ascertain the potential use of Pseudomonas spp. in settings polluted with dyes.

The findings of this research demonstrate the capacity of textile wastewater to serve as a reservoir for bacteria capable of degrading dyes. The identification of six isolates exhibiting the capacity to degrade MR and MG dyes suggests that these bacteria contain the necessary enzyme apparatus for the degradation of intricate dye molecules. The observed disparities in decolorization levels among the isolates may be attributed to variations in the chemical compositions of the dyes. Among the six bacterial isolates that were chosen, Pseudomonas spp. demonstrated the highest level of efficiency and speed in decolorizing textile dyes. This finding underscores the potential of Pseudomonas spp. in the biodegradation of hazardous dyes used in the textile industry.

The use of Pseudomonas spp. as bioagents for the degradation of dyes offers a viable and environmentally conscious strategy to address the issue of pollution caused by dye effluents. The significance of this discovery lies in the fact that textile effluents play a substantial role in water contamination, leading to detrimental impacts on aquatic ecosystems. The use of Pseudomonas spp. presents a viable, economically efficient, and ecologically sound approach to tackle this problem.

Furthermore, the present research aimed to investigate the effects of Pseudomonas spp. treated dye effluent on the development of black gramme seedlings. The findings of the study indicate that the use of treated dye effluent has a positive impact on the quality of seedlings. This is supported by the observed increase in both shoot length and weight. This finding suggests that the application of Pseudomonas spp. might have a beneficial impact on plant development, thereby alleviating the negative consequences of dye-contaminated water on agricultural yield.

CONCLUSION

In summary, the isolated bacterial strains exhibited different levels of dye decolorization, with Pseudomonas spp. displaying the highest efficacy and speed in decolorization. The use of Pseudomonas spp. as viable bioagents for the degradation of textile dyes offers a compelling alternative to traditional physical and chemical treatment approaches.

Furthermore, the assessment of seedling development demonstrated that the application of Pseudomonas spp. to the dye effluent led to an improvement in the quality of the seedlings. This finding suggests the possible use of Pseudomonas spp. in the mitigation of water pollution and the alleviation of the shortage of uncontaminated water supplies for agricultural purposes.

Nevertheless, it is crucial to acknowledge that the influence of Pseudomonas spp. on the growth of black gramme seedlings exhibited variability contingent upon the specific dye type (MR or MG) found in the effluent. It is advisable to do more study in order to get a comprehensive understanding of the underlying processes that give rise to these divergent effects. Furthermore, it is important to investigate the possible uses of Pseudomonas spp. in various settings polluted with dyes.

In general, the results of this work provide significant contributions to the understanding of sustainable approaches for managing dye effluent and indicate the potential efficacy of Pseudomonas spp. in both wastewater treatment and agricultural applications. Future research endeavours may centre on examining the potential compatibility between treated dye effluent and agricultural soils, as well as delving into the analysis of other chemical elements present in discharged textile effluents, apart from dyes.

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