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To Evaluate the Efficacy of Selected Nematode for Decolorizing Two Azo Dyes

Sunita Chauhan[1], Zinal Pankhaniya[2], Dr. Linz. Buoy. George[3], Dr. Hitesh Solanki[4]

Abstract: ***This study explores the potential of the nematode Cephalobus cubaensis to decolorize synthetic food dyes Carmacine and Ponceau 4R, emphasizing the process's efficacy and underlying biological mechanisms. Synthetic food dyes, extensively used in various industries, pose significant environmental hazards due to their toxicity, persistence, and potential to produce harmful by-products, such as aromatic amines, through conventional anaerobic treatments. Addressing these issues, biological decolorization has gained interest as an eco-friendly, sustainable alternative. Notably, C. cubaensis can degrade synthetic food dyes by releasing non-specific extracellular enzymes with potent oxidative capabilities, providing a promising pathway for sustainable dye management. This research employs spectroscopy, FTIR (Fourier-transform infrared spectroscopy), and LCMS (liquid chromatography-mass spectrometry) to analyze the degradation process and confirm the breakdown of dye molecules. These methods help elucidate the structural changes and identify the degradation products, ensuring a comprehensive understanding of the decolorization mechanism. The study underscores the importance of moving toward safer alternatives, as synthetic food dyes face regulatory restrictions due to health concerns across countries. By understanding the biological mechanisms of C. cubaensis, this research contributes to an expanding body of literature supporting biological decolorization for its environmental, cost-effective, and sustainable advantages. This is particularly relevant given the global movement toward reducing toxic chemicals in consumer products. By advancing synthetic dye treatment methods, this study promotes sustainable practices for managing dye-contaminated wastewater, highlighting the potential of nematode-based bioremediation as a viable solution.***

Keywords: synthetic food dyes, biological decolorization, *Cephalobus cubaensis*, sustainable dye management, nematode-based bioremediation

1. Introduction

Synthetic dyes, such as Carmoisine and Ponceau 4R, in food products, have become widespread due to their ability to enhance visual appeal and consumer acceptance. However, the discharge of these dyes into the environment poses significant ecological and health concerns, as they can persist and accumulate in water bodies, leading to water pollution and potential adverse effects on aquatic life and human health (Arabzadeh et al., 2014). To address this issue, the present study aimed to evaluate the efficacy of the nematode Cephalobus cubaensis in decolorizing and degrading these azo dyes in food product effluents. Azo dyes are a class of synthetic colorants characterized by one or more azo (-N=N-) groups responsible for their intense coloration. These dyes are widely used in various industries, including textile, paper, and food production. From an environmental perspective, the presence of even small concentrations of these dyes in water bodies can lead to aesthetic issues, disrupt aquatic ecosystems, and pose potential health risks due to the carcinogenic properties of their degradation products, such as aromatic amines (Sirajudheen et al., 2020). Due to their brilliant hues and inexpensive cost, synthetic azo dyes are widely utilized in various industries, including food, textiles, and cosmetics (Uppala & Muthukumaran, 2021). When released into water bodies, azo dyes, and their metabolites pose a serious risk to human health and aquatic ecosystems because they are known to be poisonous, carcinogenic, and mutagenic (Baker et al., 2011; Uppala & Muthukumaran, 2021). Azo dyes are artificial colorants widely utilized in various industries, such as food, medicine, and textiles. One or more azo bonds (–N=N–) define these dyes, which are renowned for their stability and resistance to deterioration (Singh, 2014). However, their environmental persistence is concerning because of their possible toxicity and carcinogenic effects on humans and aquatic life (Kolya & Kang, 2024).

Traditional physicochemical methods for dye removal, such as adsorption, coagulation, and chemical oxidation, often fall short due to excessive costs and secondary pollution issues. Researchers have explored various remediation techniques to address this issue, including chemical, physical, and biological methods.(Godiya & Park, 2022). Bioremediation using microorganisms has emerged as a promising and eco-friendly solution (Hu et al., 2024). Soil contamination with heavy metals from industrial and mining activities poses significant environmental and public health risks, necessitating effective remediation strategies(Haghighi Zadeh et al., 2024). Biological decolorization, utilizing microorganisms and other living organisms, offers a sustainable alternative. This research uses nematodes, microscopic worms in diverse environments, to decolorize azo dyes. Nematodes have been known to interact with bacteria, which can degrade dyes, thus potentially enhancing the decolorization process. Biological decolorization, utilizing microorganisms and other living organisms, offers a sustainable alternative.

This study investigates the potential of the *Cephalobus cubaensis* nematode, a soil-dwelling organism, to degrade and remediate the azo dye. The *Cephalobus cubaensis* nematode was selected for its ability to thrive in various environmental conditions and its potential to produce enzymes capable of breaking down complex organic compounds. To optimize the bioremediation process, we evaluated the effects of different media components and process parameters, including pH, temperature, and dye concentration, on the decolorization and degradation of azo dye by the nematode species. Our results demonstrate that the *Cephalobus cubaensis* nematode is highly effective in remediating azo dye, with decolorization rates exceeding 90%

under optimized conditions. Further analysis revealed that the nematode's enzymatic system can cleave the azo bonds in the dye molecule, forming fewer toxic metabolites.

2. Materials and Methods

2.1 Sample collection

The main objective of nematode sampling is to gather a comprehensive sample of the nematode population within a specific substrate through a systematic sampling approach. The soil sample was collected from the Mundra region near Mandavi, Kachchh district in Gujarat. Location - 22°49'51"N 69°40'55"E.

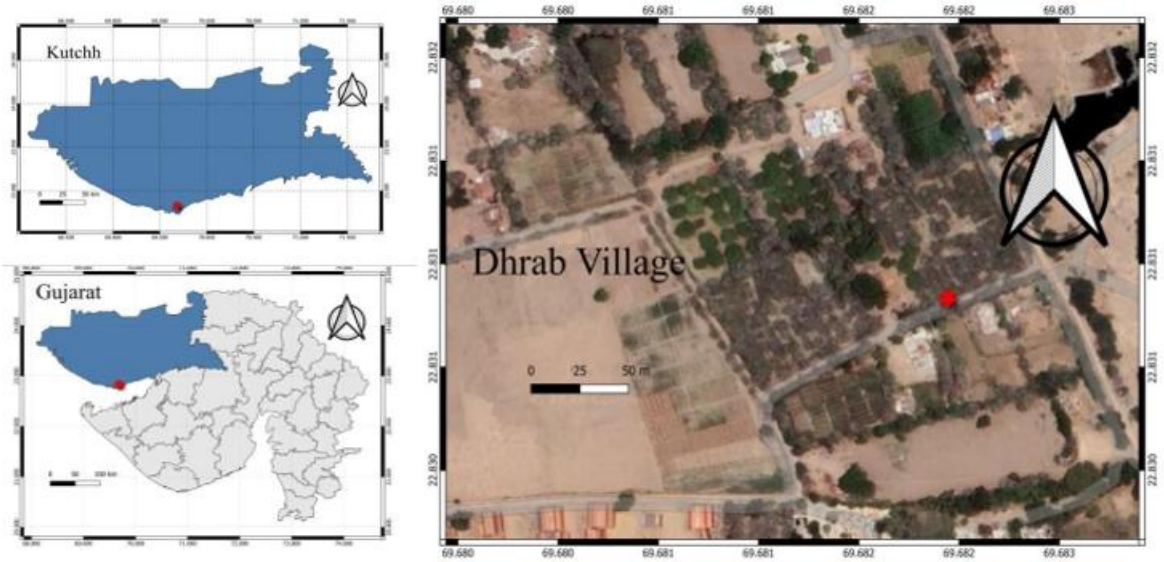

Figure 2.1: Selection of site Source: QGIS- 3.36.1

2.2 Nematode Extraction

We (Haghighizadeh et al., 2024)(Lewis, et al.,1995). Deposit the encapsulated substrate into a petri dish equipped with a mesh base. Introduce an aqueous solution until it marginally surpasses the mesh level, ensuring the encapsulated substrate remains in hydric contact. Allow the system to equilibrate for a period ranging from 24 to 72 hours. Maintain a consistent hydric interface by covering the dish, thus mitigating desiccation. After the incubation, extricate the substrate package and employ a stereoscopic microscope to examine the aqueous medium for nematode presence. This methodology leverages the hydrostatic behavior of nematodes to facilitate their migration from the substrate into an observable medium. The nematodes that had migrated into the aqueous medium were subsequently isolated via centrifugal processing.

2.3 Cultivation

The species' adult nematodes were randomly selected and transferred to agar plates. The agar medium composition was as follows (per 100 ml): 4 g of agar, 0.13 g of peptone, 0.2 g of NaCl, supplemented with 5 ml of nutrient water, 1 ml of Mgs_4, 1 ml of $CaCl_2$, and 1.5 ml of boric buffer. This method was originally Lewis's, but the author modified using a boric buffer (Lewis et al.,1995). The cultures were incubated at a constant temperature of 27°C. Observations were conducted every week for up to one week to ascertain the establishment of cultures by the nematodes. An antimycotic antibacterial solution was used to mitigate the transfer of bacterial and fungal contaminants from the soil.

Post-incubation, aqueous samples from the Petri dishes were transferred to centrifuge tubes and subjected to centrifugation at 3500 revolutions per minute (rpm) for 3 minutes. Subsequently, the resultant sediment was carefully collected onto Petri plates. The presence of nematodes was then verified through examination under a Nikon (Japan) stereoscopic microscope.

2.4 Identification

Morphological and morphometric image analysis was performed with extracted nematodes by transferring the nematode pellet into the cavity slides. After that, slides were visualized with a digital light microscope (Panthera L with built-in image system), and all morphological observations were recorded with inbuilt microscope software (motion image) in the form of images for further analysis. In addition, measurements of the body, tail, and mouth were recorded with the help of image analysis software ImageJ.

The phylogenetic analysis used partial sequences of the 18S ribosomal RNA gene to elucidate their evolutionary relationships.

2.5 Decolourizing Assay

Entomopathogenic nematodes are prepared for encapsulation in a concentrated suspension, usually ranging from one thousand nematodes per 0.1 ml. They are cultured in NGM media under controlled conditions, maintaining a temperature of 24°C and a pH of 8.5, to ensure their viability before encapsulation in the laboratory, the survival and activity of the encapsulated nematodes are evaluated, and their release rates from the beads are adjusted to match the conditions of the dye-contaminated environment The mixture is then dripped into a calcium chloride solution, triggering the gelling of alginate and encapsulating the nematodes within the beads. These beads are allowed to harden to achieve the necessary consistency and durability. These nematodes are then embedded within calcium-alginate beads, a gel-like safeguarding them from desiccation and sunlight. During encapsulation, the nematode population is mixed into the beads with a dye solution at a concentration of 20mg per 100 ml with even distribution for effective treatment. The remediation process is monitored by measuring the optical density of the solution to track dye degradation. After a 12-day remediation period, the solution is analyzed to determine the level of dye degradation. This method demonstrates a controlled approach to bioremediation, utilizing nematodes encapsulated in sodium alginate beads to effectively break down food dyes in contaminated settings.

2.6 UV-Visible Spectroscopy

We are using a Lab-India UV-visible spectrophotometer with UV-VIS software. In this, we plotted absorption peaks for Sunset yellow dye at the 485nm wavelengths. The absorbance of dye triplicate average of control and sample for 24 24-hour period time with 12 days of treatment is obtained as a graph, and the change in peaks at the initial and post-treatment stages was compared. Spectra peaks were plotted and compared.

2.7 FTIR Spectroscopy

Fourier Transform Infrared Spectroscopy, or FTIR, is a potent analytical method that is used to detect chemical substances and analyze their vibrational properties. This absorption produces a fifty-nine distinctive infrared spectrum that can be utilized to determine the sample's chemical composition (Nandiyanto et at., 2019). FTIR analysis was performed by the Shimazdzu IRSpritit instrument using LabSolutions IR software to characterize the existence and accountability of functional groups present in the Dyes. FTIR spectra of the samples before and after the experiment were obtained using the FTIR spectrum. Data were collected within the mid-infrared region from 4500 to 500 cm^{-1}.

2.8 LC-MS Analysis

After a 12-day treatment, an LC-MS analysis was directed to demonstrate the separation of a compound from the main components of sunset yellow. In LC-MS analysis we use a Thermos Scientific Mass Spectrometer, LCQ Fleet model using LCQ Fleet tune software. This analysis was performed on a C18 Column. The mobile phase was made up of solvent A, ammonium acetate at pH 7.8 (adjusted with ammonia solution 2.5%), and Solvent B acetonitrile. The gradient used was after isocratic conditions as mobile phase A for 0-3 mint 98-50%B,3-8 mint 50-0%B,8-15 mint 0%B 15-15.5mint 0-98%B 15.5-20mint 98%B (For Carmosine) A: B:100:0 V/V to A: B:40:60 V/V (Ponceau 4R). The respective flow rate was 0.4mL/min and 1mL/min at 50°C±1°C, and 10 and 3µL of the extract was injected into the column. The flow was directed into the MS detector for 20 min.

3. Results and Discussion

The soil samples, mixed with water, are examined under a stereomicroscope to detect the presence of nematodes. The nematode extracted from soil samples showed a positive outcome in terms of survival rate. Since the presence of nematodes in the soil samples was confirmed, the samples are now being directed toward nematode identification. The identified nematode species is *Cephalobus cubaensis.*

3.1Morphological Identification

C*ephalobus cubaensis* is a free-living, soil, bacterivorous, and fungivores nematode first found from roots of Mattu Gulla, a unique Brinjal variety grown in Mattu village, Udapi district of Karnataka, India (Muthusamy, 2019).

3.2 Characteristics

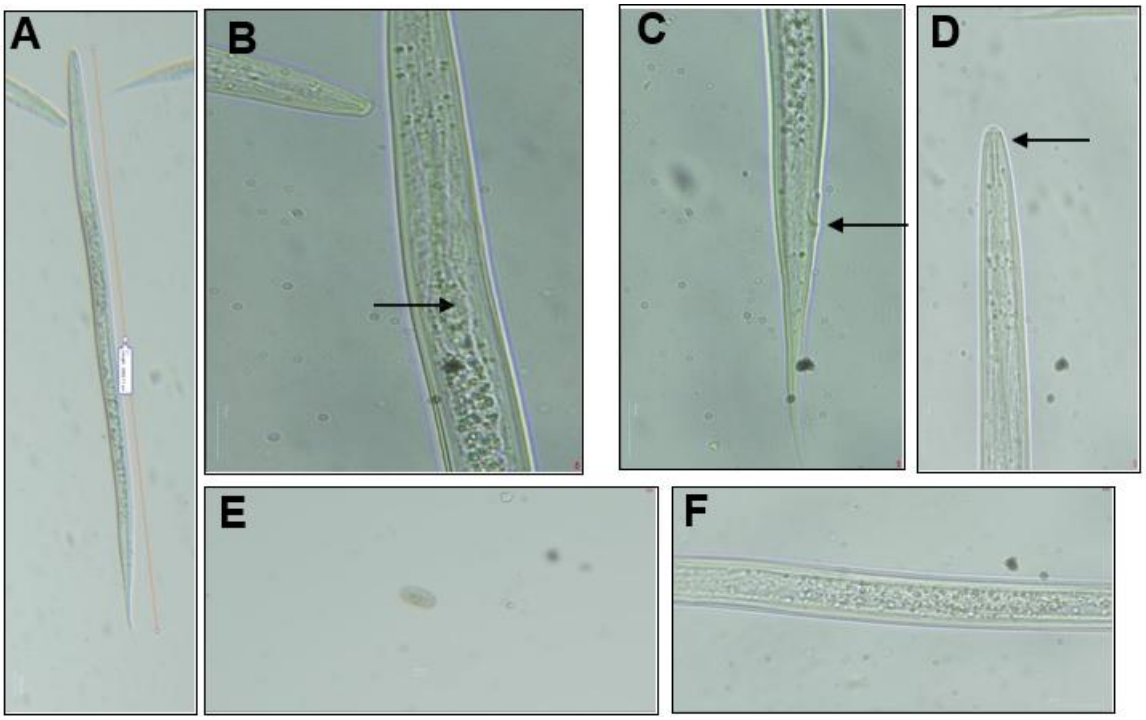

Figure 3.1: *Cephalobus sp. (*A) Adult female (B) Oesophageal bulb (C) Tail and anus (D) Mouth part (E) Egg (F) Mid region of the body.

The nematodes are small, ranging from 0.45mm to 0.70mm in length, with slightly tapered bodies. Their heads are somewhat lobed, and they lack a caudal sucker. The cuticle is annulated, with annuli about 1.5 µm wide at the midbody, and features well-marked transverse striae but no setae or papillae. The uterus is asymmetrical, and the spicules are slightly curved and fusiform, with a recognizable posterior median accessory piece. The male tail may be pointed. The lip region lacks probate, and the nerve ring typically surrounds the base of the corpus anterior.

Nematode species identification, once based solely on morphology, is now enhanced with molecular techniques. This approach provides greater accuracy and certainty, complementing traditional methods and offering a more comprehensive understanding of nematode taxonomy and diversity.

3.3 Molecular identification of *Cephalobus cubaensis*

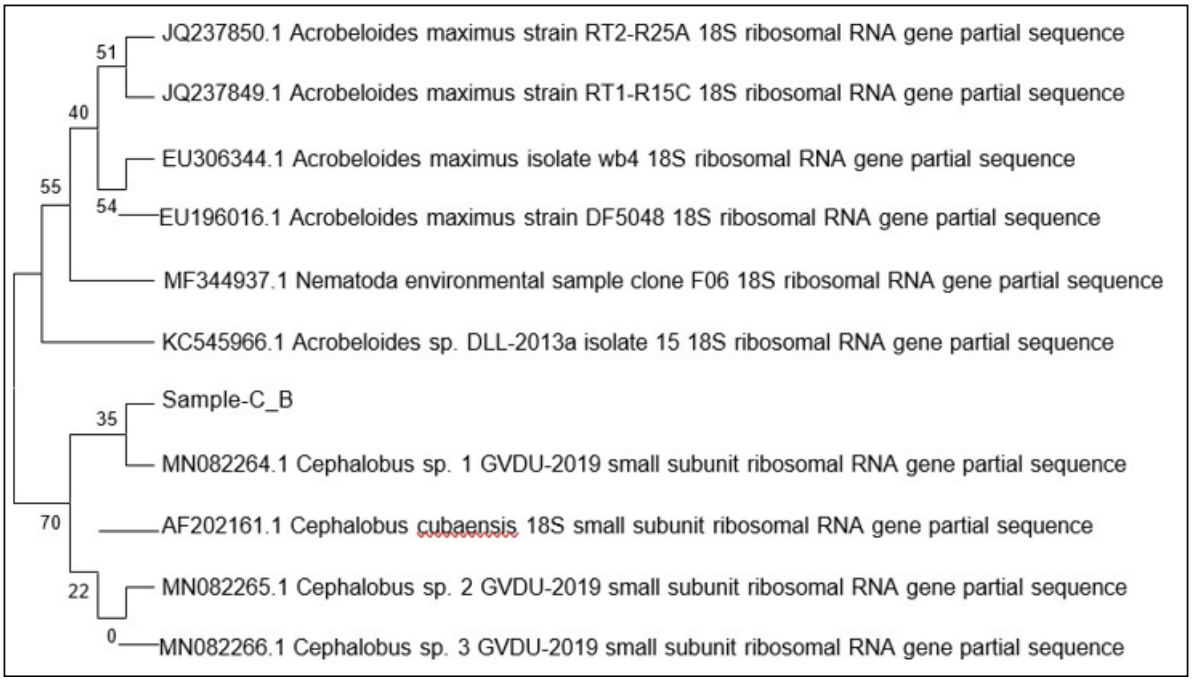

Figure 3.2: Phylogenetic tree of *Cephalobus cubaensis*

The identification results indicate that the *Cocos nucifera* sample shares approximately 99% nucleotide homology with *Cephalobus cubaensis* based on phylogenetic analysis. Consequently, *Cephalobus cubaensis* was chosen to conduct the decolorization assay.

3.4 Analysis of dye decolorization experiment:

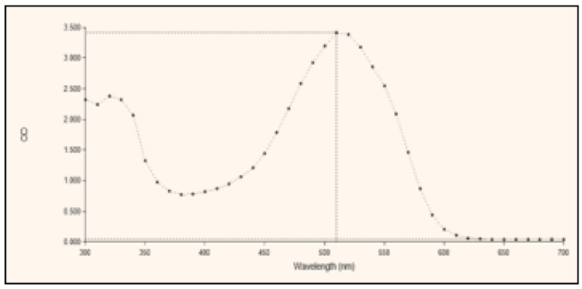

Figure 3.3: Lambda max of Carmoisine

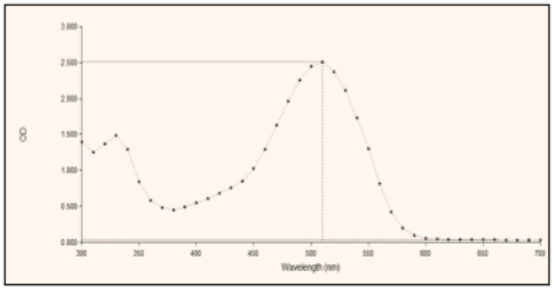

Figure 3.4: Lambda max of Ponceau 4R.

At a concentration of 1 mg/100 ml, the lambda max of Carmoisine and Ponceau 4R is 516nm and 505nm, respectively.

3.5 UV visible spectroscopic analysis

In this experiment, 30mg/100 concentrated samples of Carmoisine and Ponceau 4R dyes were used, with absorbance measured at 516nm and 505nm, respectively. Over time, a decrease in absorbance was observed for both dyes. Graphs showed the correlation between absorbance and wavelength for both dyes, comparing control samples (untreated) and treated samples (exposed to nematodes) after 15 days. The treated dyes showed a decrease in absorbance, while the control samples' absorbance remained steady.

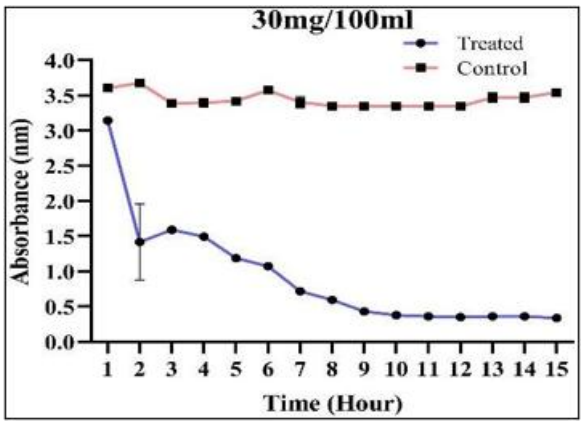

Figure 3.5: Graphs showing variation in absorbance of Carmoisine dye: Control VS Treated (30mg/100ml)

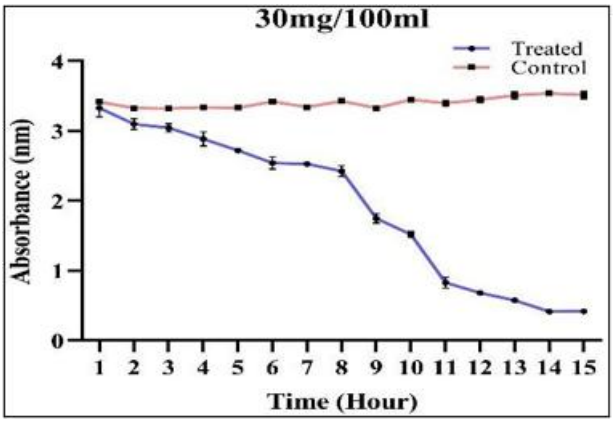

Figure 3.6: Graphs showing variation in absorbance of Ponceau 4R dye: Control VS Treated (30mg/100ml)

After 15 days of treatment, UV-visible spectroscopy analysis revealed a consistent reduction in Carmoisine dye absorbance when treated with nematodes encapsulated in sodium alginate beads. Conversely, the control sample showed no notable change in dye absorbance. This suggests that the treatment process involving nematodes within sodium alginate beads gradually diminishes the presence of Carmoisine dye, showing a potential application for dye degradation or removal processes.

The average of the triplicate measurements for each dye was 15 days decolorization data is used to calculate, the mean, standard deviation, and standard error, and p-values were determined using GraphPad Prism software. The p-values obtained were 0.4 for Carmoisine and 0.3 for Ponceau 4R, indicating a significant change over time.

3.6 FTIR analysis

The UV spectroscopy results were confirmed by FTIR, which showed significant changes in the vibrational frequencies of Carmoisine and Ponceau 4R dyes after fifteen days of treatment with immobilized nematodes, compared to the control group. The FTIR analysis of Carmoisine after treatment revealed noticeable changes in the functional groups, with various stretching and vibrational frequencies detected. The peaks at 3324.8 cm^{-1} (-N-H), 2227.1 cm^{-1} (C≡C), 2158.1 cm^{-1} (-SCN), 2137.6 cm^{-1} (-NCS), 1960.6 cm^{-1} (C-H), 1638.2 cm^{-1} (C=C), 1067.9 cm^{-1} (Aromatic C-H), 978. cm^{-1}, 752.9 cm^{-1} (C-H), and 663.5 cm^{-1} (C-H bend) are shown in the control (not treated with nematodes), while peaks at 3302.4 cm^{-1} (H bonded -OH stretch), 2519.7 cm^{-1} (C-H), 2156.3 cm^{-1} (-SCN), 2119.0 cm^{-1} (-NCS), 2010.9 cm^{-1} (C=C), 1913.6 cm^{-1} (C-O), 1880.4 cm^{-1} (C≡N), 1636.3 cm^{-1} (C=C), 1067.3 cm^{-1} (Aromatic -CH) are shown in the sample treated with the nematodes. FTIR analysis reveals significant changes in the chemical composition of Ponceau 4R after treatment. The control peaks at 3277 cm^{-1} (hydroxy group -H bonded -OH stretch) are replaced by peaks at 3325 cm^{-1} (aliphatic secondary amine -N-H stretch). Additionally, a common peak in both the control and treated groups at 1635.70 cm^{-1} shifts to 1635.45 cm^{-1}, indicating a C=C aromatic stretch. These changes in vibrational frequencies detected by FTIR analysis demonstrate alterations in the dye's structure.

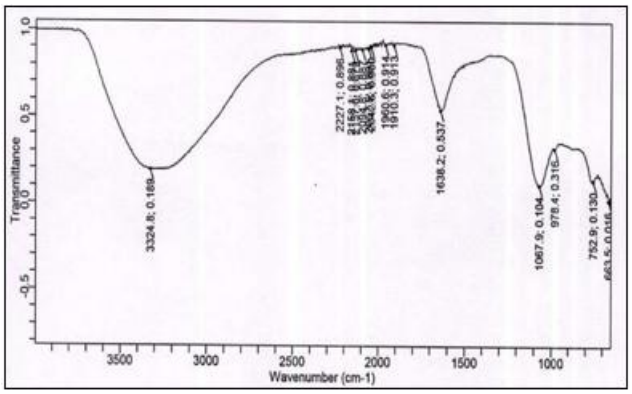

Figure 3.7: Before treatment -Carmacine

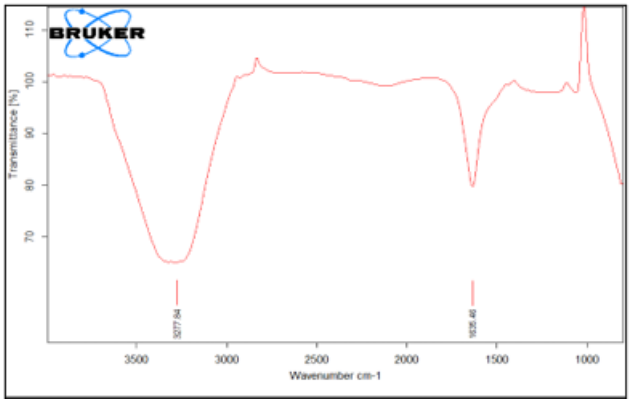

Figure 3.9: Before treatment-Ponceau 4R

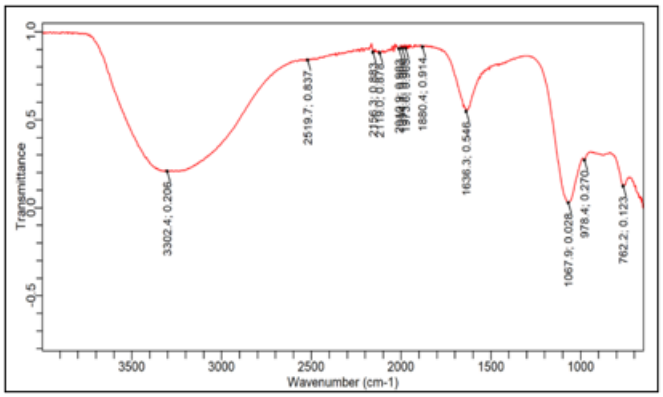

Figure 3.8: After treatment- Carmacine

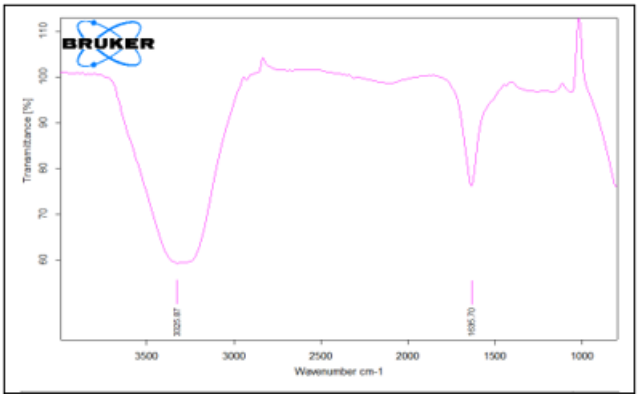

Figure 3.10: After treatment-Ponceau 4R

3.7 LC-MS Analysis

The FTIR analysis of Carmoisine and Ponceau 4R reveals significant changes in the compounds present in both dyes. These changes are further verified by LC-MS analysis.

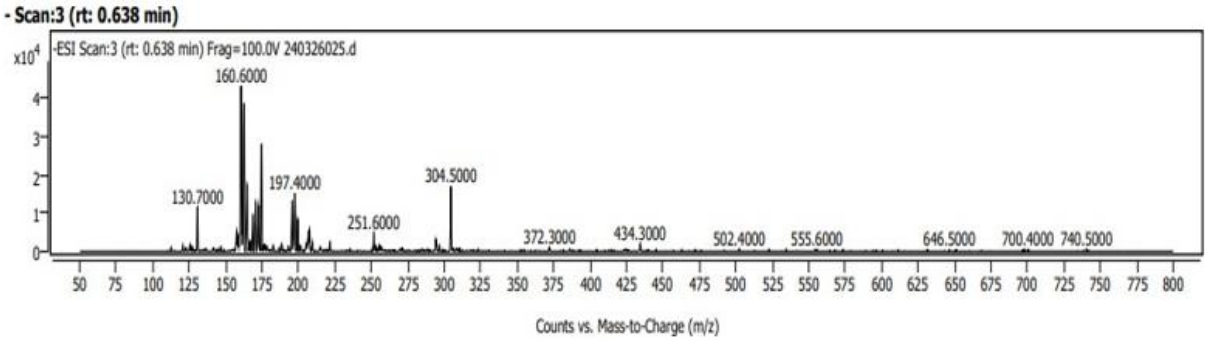

Figure 3.11: LC-MS analysis of Carmoisine – Before treatment

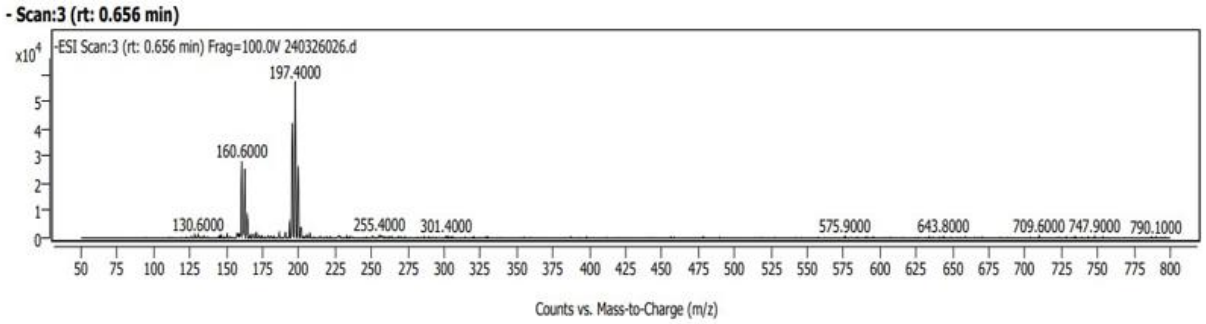

Figure 3.12: LC-MS analysis of Carmoisine – After treatment

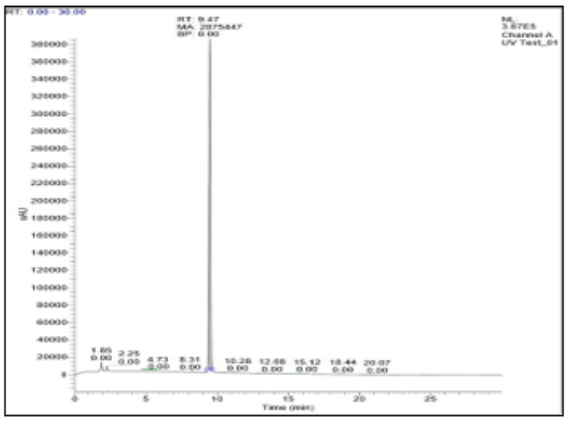

Figure 3.13: LC-MS analysis of Ponceau 4R–Before treatment

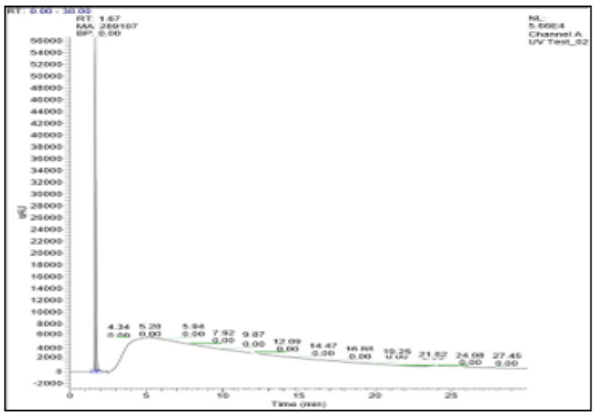

Figure 3.14: LC-MS analysis of Ponceau 4R–After treatment

The LC-MS analysis of the Carmoisine dye post-treatment revealed a complete disappearance of the molecular ion with a weight of 502.44, which was initially present in the control group. This shows a profound impact of the nematode treatment on the dye, leading to its degradation or transformation into different compounds not identifiable in the given LC-MS conditions.

The LC-MS data revealed a significant shift in the retention time (RT) of the Ponceau 4R dye when treated with nematode-encapsulated sodium alginate beads. In the control sample, the dye showed a peak at an RT of 9.43 in positive mode with the highest abundance. Contrastingly, the treated sample displayed a marked RT shift from 9.43 to 1.47, accompanied by a decrease in abundance to sixty. This suggests a substantial alteration in the dye's chemical structure post-treatment.

4. Discussion

Human activities transforming natural land into agricultural areas reduce the diversity of plant and animal life above ground (Foley et al., 2005). Nematodes, abundant in agricultural environments, play a significant role in soil biodiversity across large regions. These soil-dwelling organisms are classified into five trophic groups based on their functions: microbivores, fungivores, plant parasites, predators, and omnivores (Wasilewska, 1971; Yeates et al., 1993). This study collected soil samples from coconut tree roots and found that nematodes, due to their stress response mechanisms, are easy to maintain in the lab as they tolerate changes in pH, temperature, and oxygen levels (Mobjerg et al., 2011). Many nematode species are either undiscovered or poorly understood, with identification methods ranging from traditional morphology to advanced high-throughput sequencing, each with its pros and cons (Seesao et al., 2016). Using both morphological and molecular techniques, this study identified *Cephalobus cubaensis*, a free-living, soil-dwelling, bacterivorous, and fungivores nematode first found in the roots of Mattu Gulla, a unique brinjal variety from Karnataka, India (Muthusamy, 2019).

Water pollution from dye industries is a significant environmental issue. Annually, about 106 tons of azo dyes are produced globally, with 1-1.5 × 10^5 tons released into the environment via wastewater (Zollinger, 2003). These dyes, used in various industries, are toxic depending on their chemical structure. Synthetic azo dyes are persistent and pose ecological challenges due to their carcinogenic potential and resistance to degradation. To address this, various techniques, including chemical, physical, and biological methods, have been developed for dye degradation, with biological methods often preferred for their eco-friendliness. Various microorganisms have shown effectiveness in dye decolorization. This study used *Cephalobus cubaensis* to decolorize two food azo dyes, Carmoisine, and Ponceau 4R, by immobilizing them in sodium alginate beads and analyzing the results with UV-visible spectroscopy, FTIR, and LC-MS.

UV-visible spectroscopy showed a decrease in absorbance for both dyes after 15 days of treatment, indicating effective decolorization. FTIR analysis revealed changes in functional groups, such as the transformation of the -N-H bond to the -OH group and the formation of a C≡N bond. LC-MS analysis showed the absence of the original molecular weight peak for Carmoisine and significant RT shifts for Ponceau 4R, indicating the breakdown of dye structures and the formation of non-toxic compounds. Overall, treatment with *Cephalobus cubaensis* led to significant molecular changes in the dyes, suggesting that these nematodes possess enzymatic systems capable of effectively degrading and decolorizing azo dyes.

5. Conclusion

This study demonstrates that *Cephalobus cubaensis* effectively degrades the synthetic food dyes Carmoisine and Ponceau 4R. Through a combination of UV-spectroscopy, FTIR, and LC-MS analyses, the research highlights the nematode's potential as a bio-decolorization agent. UV-spectroscopy revealed a significant decline in absorbance after 15 days of treatment, indicating effective dye breakdown. FTIR analysis showed alterations in stretching and vibrational frequencies, suggesting a bond breakdown in the dye molecules. LC-MS analysis detected significant shifts in retention time (RT) and a decrease in abundance, confirming enzymatic activity leading to the degradation of the dyes' molecular structures.

These results suggest that *Cephalobus cubaensis* can transform harmful dyes into less toxic intermediates, making it a promising candidate for sustainable wastewater treatment. The findings underscore the importance of eliminating toxic

dyes from wastewater to mitigate environmental contamination. Utilizing *Cephalobus cubaensis* in bioremediation efforts can advance eco-friendly and sustainable wastewater treatment methods, contributing significantly to environmental preservation.

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