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Research paper

Phytoremediation of mixed reactive Azo dyes in contaminated water by *Ceratophylum demersum* and its toxicity analysis on other life forms

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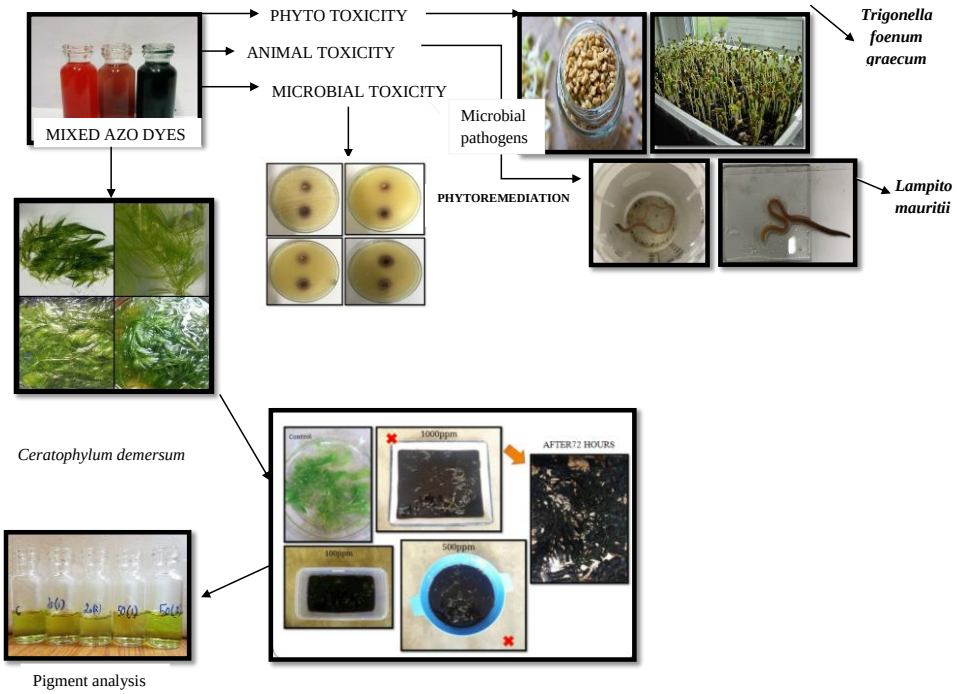
Abstract

Textile dyes are toxic in nature as they exert various harmful effects on living organisms. These effluents are the main source pollutant to various ecosystems, especially to soil. In this study, mixed azo dye from a local fabric dyeing industry were analysed for their toxicity effects on *Trigonella foenum graecum*, *Lampito mauritii*, and bacterial pathogens. The results presented in the study showed that the toxicity of the mixed azo dye prompted *Trigonella foenum graecum* root and shoot growth inhibition on 1000 ppm of mixed azo dye contaminated soil, and this effect decreased when performed on remediated soils with higher concentrations of the effluents. Phytoremediation of water contaminated with different concentrations of mixed azo dye solutions was performed using *Ceratophylum demersum* where, the dye removal percentage was minimal and the plant couldn't sustain more than 500 ppm of dye. Dye toxicity on earth worms showed increased percentage of protein content of the body in response to stress (azo dye). These results revealed that the textile dyes we toxic to eukaryotic cells and that can potentially lead to adverse health conditions. Further their toxicity was also tested against earthworms and pathogenic bacterial strains.

Keywords: *Trigonella Foenum Graecum; Lampito Mauritii; Ceratophylum Demersum; Phytoremediation; Textile Dyes; Mixed AZO Dye.*

Graphical abstract



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1. Introduction

Textile industries contribute nearly 14% of the total industrial production in India and discharge large volume of effluent after dying process (Ekambaram et al., 2016). Textile effluent is generated through printing, dying, bleaching, sourcing and mercerizing processes. Effluent from textile industries contains cotton, wool, silk, synthetic fibers, coloring agents (synthetic dyes), bleaching agents (chlorine), fixing agents (formaldehyde, benzidine etc.), stain removing agents and printing gums (Ponraj et al., 2011). Azo dyes are largest group of dyes that consists of 70% of dyes used in textile industries and produces clear and strong colors (Jadhav et al., 2016). These dyes are xenobiotic compounds characterized by the presence of one or more azo linkages and aromatic rings. They are the largest class of dyes with greatest variety of colors (Madhuri et al., 2014). Removal of colors from textile effluent is one of the major environmental concerns. Degradation and decolorization can be achieved through physical, chemical and biological methods. Physical and chemical methods are generally expensive as compared to biological methods and produce a large amount of secondary waste.

Some investigators reported that the azo dyes and their metabolites are toxic, carcinogenic, and mutagenic in nature which leads to the formation of tumors and allergies besides growth inhibition of bacteria, protozoan, algae, plants and different animals (Saratale et al., 2014). They are light-absorbing compounds and significantly affect the photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Hemapriya, 2013). When high concentration dye-containing water is used for washing and bathing purposes, it may cause several skin diseases like ulceration of the skin, dermatitis, respiratory tract infections, perforation of nasal sputum and ingestion may cause vomiting, pain, hemorrhage and sharp diarrhea (Iqbal and Nisar 2015). There are various ways in which plants are used to remediate organic compounds from contaminated sites. To remove the recalcitrant compounds from soil, sediment and/or water, plants can break down, or degrade organic pollutants.by the process of phytoremediation.

Phytoremediation of textile dyes is reasonably a new method of textile effluent treatment. Various strategies for laboratory scale studies have been employed. Studies on textile dye removal have mostly been focused on the use of wild macrophytes. *P. australis* was explored for the removal of Acid Orange 7 as the model dye (Kolikar et al., 2008). Narrow-leaved cattail (*T. angustifolia*) showed the potential in treatment of Reactive Red 41 at concentrations of 100-300 mg/L and could achieve around 60% decolorization. Lemna minor was also proposed to remove Methylene Blue dye with accumulation to be the prominent mechanism (Umbuzeiro et al., 2005). Aquatic plants like *Eichhornia* spp. were also found to remove Direct Dark Blue 6B, Black HY and Congo red (Charumathi, 2010). A narrow range of herbaceous plant species is known to perform the removal of harmful dyes. *Z. angustifolia, Brassica juncea, B. malcolmii, T. patula, T. flagelliforme, A. amellus, G. pulchella, Sesuviumportulacastrum, Gaillardia grandiflora, Rosmarinusofficinalis, Petunia grandiflora, L. minor, Azollafilicu-loides, Portulaca grandiflora, Thymus vulgaris, I. hederifolia and Hydrocotylevulgarisare* some of the examples which have been proposed for the removal of textile effluents and dyes (Torbati et al., 2014). This present study was attempted to study on the remediation of mixed azodyes using the *Trigonella foenum graecum and Ceratophylum demersum*. The mixed azo-dyes toxicity was also tested against earthworms and pathogenic bacterial strains.

2. Materials and methods

2.1. Dyes and chemicals

The textile dyes (C.I. Reactive red (RR), C.I. Reactive brown (RB), C.I. Reactive black (RBK) were purchased from textile industry.

2.2. Phytotoxicity studies

Phytotoxicity tests were performed by modifying the protocol given by Durve et al., (2012). In order to assess the toxicity of the untreated and treated soil samples the phytotoxicity tests were carried out on fenugreek seeds (*Trigonella foenum graecum*). About 20 healthy plant seeds were treated separately with 1000 ppm of each of individual dyes. The same is followed with the remediated soil, as well as healthy soil is kept as control. All the experimental sets were watered with 5 ml of water for 7 days. Germination percentage as well as the length of plumule and radical was recorded after 7 days. The experimental set up is given in table 1.

Table 1: Experimental Set Up for Analyzing Azo Dye Toxixcity on *Trigonella Foenum Graecum*

Experimental Set Up	
Control	Pot With Healthy Soil
Negative Control	1000 Ppm Pot
Pot C1 & C2	Treated With Free Cells Of Bacillus Cereus
Pot D1 &D2	Treated With Free Cells Of Enterobacter Cloacae
Pot E1 &E2	Treated With Co-Culture
Pot B&C	Treated With Encapsulated Co-Culture

2.3. Earthworm toxicity

Earthworm is commonly found in the upper layer of the earth, up to the depth of 30–50 cm from the surface. The suitable appropriate time for the collection of earthworm was found to be early morning in the summer and noon time during the winter. The collected live earthworms were stored in the plastic bags filled with wet compost soil. Earthworms were placed at 25˚C in the laboratory conditions.

The experiments were carried out as prescribed in the Organization for Economic Cooperation and Development test protocol (Rombke and Moser., 1999). Prior to the use of earthworm for experimental work they were acclimatized for seven days under the laboratory conditions. A wide mouth jar covered with a muslin cloth having moist soil compost manure was used as a feed medium for the earthworms. Adult earthworms weighing 300–600 mg having clitella were used in experiments.

2.4. Preparation of the test compound

Stock solutions of were prepared having a concentration of 25,000 mg/L. From this stock solutions four different concentrations 100, 250, 500, 400 mg/L were prepared for mixed reactive azo dyes (RR, RB & RBK).

2.5. Method for treatment

Contact feeding method was used for the exposure of the earthworms to the untreated dye. Three air-dried mud pots containing 500g of soil were amended with suitable dose of the untreated and decolorized solutions of RR, RB, RBK.
The pots were marked for their respective concentration, whereas one pot was kept as a reference to determine the environmental effect. Five adult earthworms of same age and size were released in each of the beakers. At regular time intervals, weight of the earthworms and morphological as well as pathological symptoms were monitored. Percent mortality was determined on the basis of data obtained on earthworms exposed to untreated dye solutions. If mortality was found to be more than 20% in the control set, then the entire experiment was repeated.

2.6. Determination of the total protein content

The effect RR, RB, RBK dye solutions was studied on the total protein content of the earthworms. Five adult earthworms were exposed dye solutions. Thereafter, the exposed earthworm's clitellum, head, and abdomen were removed and suspended in 2ml of deionized water and homogenized for 15min. The homogenate thus obtained was centrifuged at 5,000 g for 10min at 4˚C and the supernatant was used for the estimation of protein.

2.7. Microbial toxicity

The microbial toxicity of the dye solutions was determined against test microbes such as *Klebsiella pneumonia, Proteus mirabilis, Staphylococuss aureus, Escherichia coli*. Microbial toxicity study was determined by agar well diffusion assay. Actively grown micro-organisms were seeded on the nutrient agar plates. Four wells of 8mm diameter each were bored in nutrient agar media. The wells were filled with 100 and 200 ppm of mixed reactive azo dye solutions of. The plates were incubated at 30˚C for 48h and zone of inhibition (in mm) was measured representing the index of the toxicity.

2.8. Collection of plant material and generation of plant stock for decolorization studies

Plants of Ceratophylum demersum were collected from local aquarium plants seller, and were allowed to grow in the water tubs. A stock of the plant was produced by placing them in normal tap water for one month to achieve vigorous and dense shoot system to be used further for in vitro dye. Decolorization experiments were initially carried out with the wild plants using the mixed dyes (C.I. Reactive red, C.I. Reactive brown, C.I. Reactive black).
The experiments were carried out in 500 mL rectangular containers having 200 mL of 20,50,100,500,1000 mg/L of dye solution respectively in plain distilled water. Absorbance of dye solution was recorded in an interval of 24 h each by removing 3 mL of solution. This solution was centrifuged at 4561 g for 10 min (Khandare et al., 2012) to remove any solid residue from plant if present, and the absorbance of the clear solution was measured at the wavelength of 369 nm and decolorization percentage was calculated.

2.9. Photosynthetic pigments analysis

Plants contain two types of pigments chlorophylls (chlorophyll a and b) and carotenoids (carotenes and xanthophylls). 0.20 g leaves (major veins and fibrous tissues were removed) of both control and test plants were taken in mortar and pestle, 20 mL of acetone was added and crushed, while crushing a pinch of $MgCO_3$ powder was added. The formed homogenate was filtered and centrifuged for 10 min at 2000 g. Chlorophyll a, chlorophyll b and carotenoids were determined spectrophotometrically taking 80% acetone as a blank. Chlorophyll content was measured by taking readings at 645 and 663 nm. Carotenoid content was estimated at the wavelength of 470 nm and Arnon's equations were used to convert absorbance measurements to mg Chl/g leaf tissue (Arnon, 1949).

- Total chlorophyll

Chl_a (mg/g) =[(12.7 A_{663}) (2.6 A_{645})] ml acetone/mg leaf tissue
Chl_b (mg/g) = [(22.9 A_{645}) (4.68 A_{663})] ml acetone/mg leaf tissue

- Carotenoids and Xanthophylls

$C_{xþc}$ =1000 A_{470} 1.90Chl_a 63.14 Chl_b/214, (x ¼ xanthophylls and carotenes)

Chl_a - Chlorophyll a
Chl_b- Chlorophyll b
$C_{xþc}$- Carotenoids and Xanthophylls
A_{663}- Absorbance at 663 nm
A_{645}- Absorbance at 663 nm
A_{470}- Absorbance at 663 nm

3. Results

3.1. Phytotoxicity analysis in the mixed azo dye degraded soil samples by free cells

In various pots, 10 seedlings were planted. Pot A contained fertile soil which had 9 sproutings at the end of 7th day and the average root and shoot length were found to be 9.4 cm and 8.2 cm respectively. Pot C1 contained mixed reactive azo dyes degraded soil by Enterobacter cloacae, which had 6 sprouting at the end of 7th day and the average root and shoot length was found to be 2.8 cm and 3.5 cm respectively. Pot D1 and D2 contained mixed reactive azo dyes degraded soil by Bacillus cereus which had 6 sproutings at the end of 7th day and the average root and shoot length were found to be 3.7 cm and 3.6 cm respectively. Pot E1 and E2 contained mixed reactive azo dyes degraded soil by co-culture which had 8 sprouting at the end of 7th day and the average root and shoot length varied from 4.5 cm and 5.7 cm respectively. The pot contaminated with 1000 ppm of mixed reactive azo dyes showed only 3 sproutings at the end of 7th day and the average root and shoot length were determined to be 1.9 cm and 2.9 cm respectively, which showed very low growth rate when compared with other degraded pots with Enterobacter cloacae and Bacillus cereus. Figure 1-9 shows the growth of *Trigonella foenum gracum* in each of the pots.

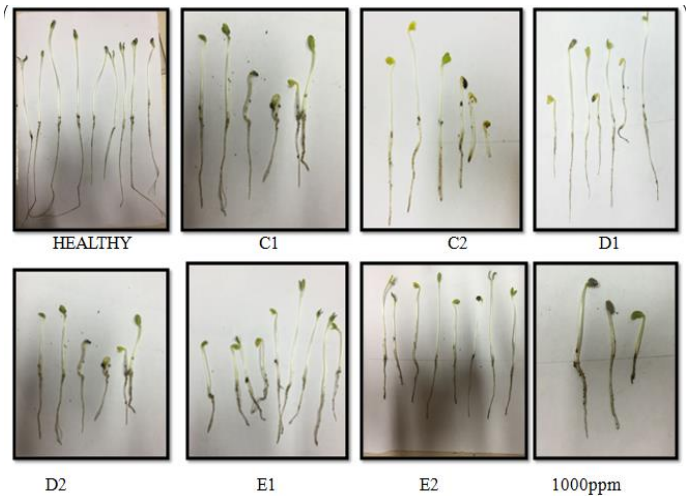

Fig. 1: Growth of *Trigonella Foenum Graecum* in Each of the Free Cell Pots.

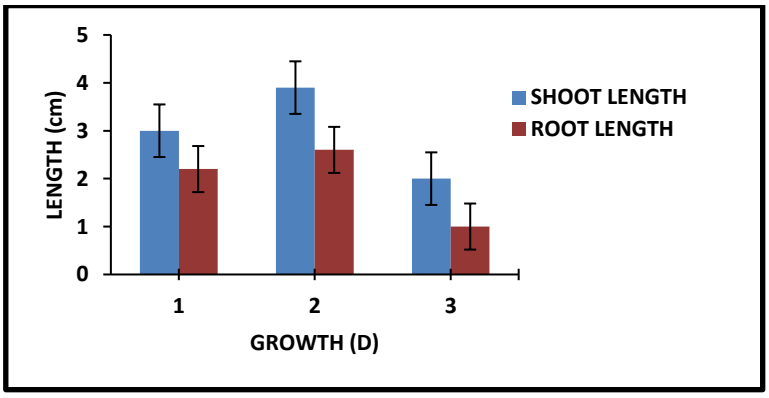

Fig. 2: Growth of Trigonella Foenum Graecum in 1000 Ppm.

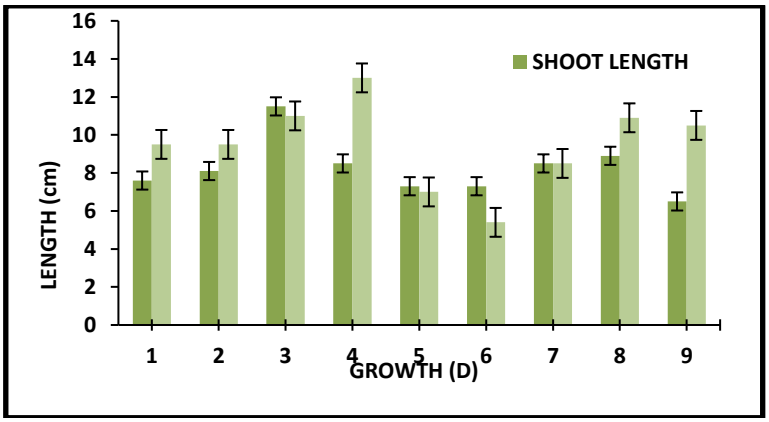

Fig. 3: Growth of Trigonella Foenum Graecum in Control.

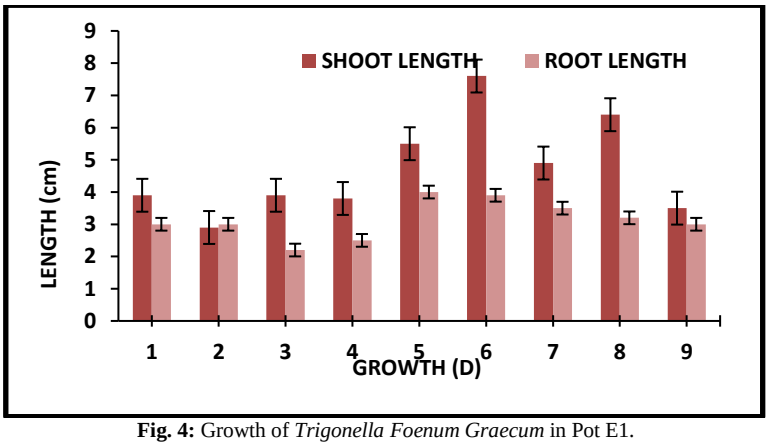

Fig. 4: Growth of *Trigonella Foenum Graecum* in Pot E1.

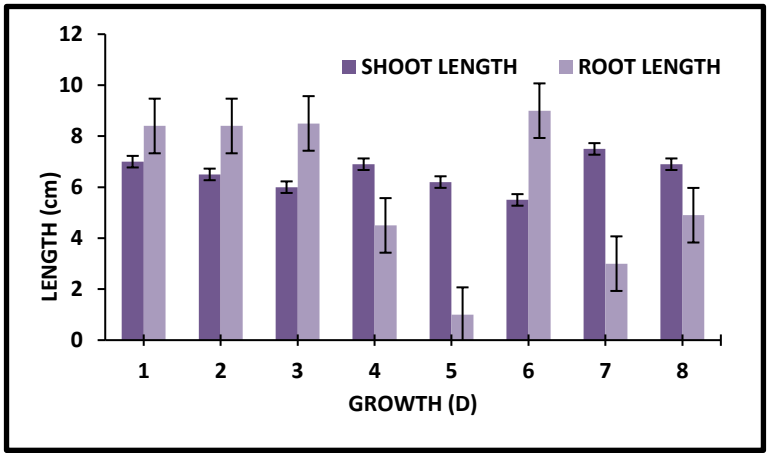

Fig. 5: Growth of Trigonella Foenum Graecum in Pot E2.

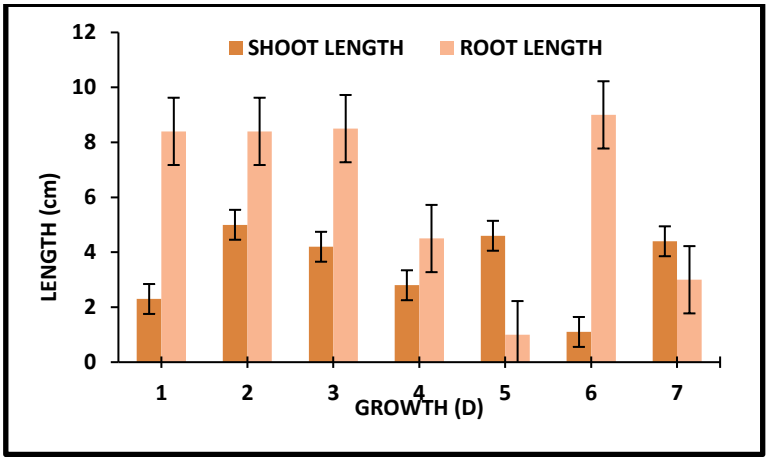

Fig. 6: Growth of Trigonella Foenum Graecum in Pot D1.

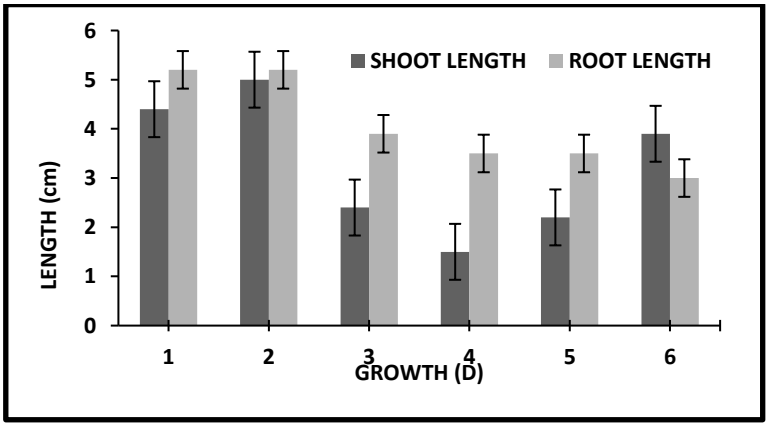

Fig. 7: Growth of Trigonella Foenum Graecum in Pot D2.

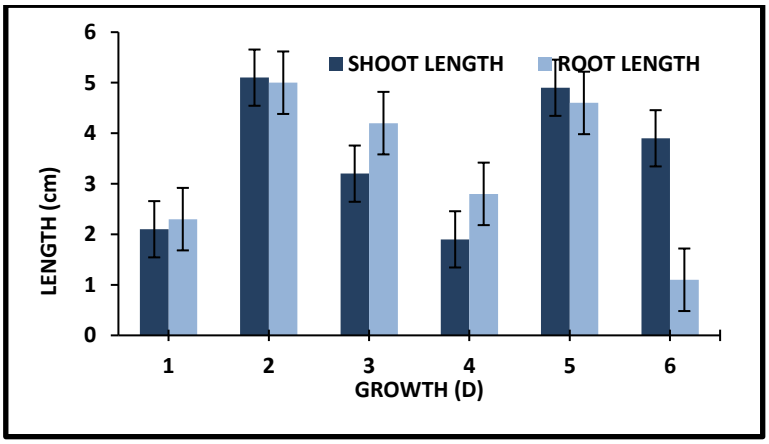

Fig. 8: Growth of Trigonella Foenum Graecum in Pot C1.

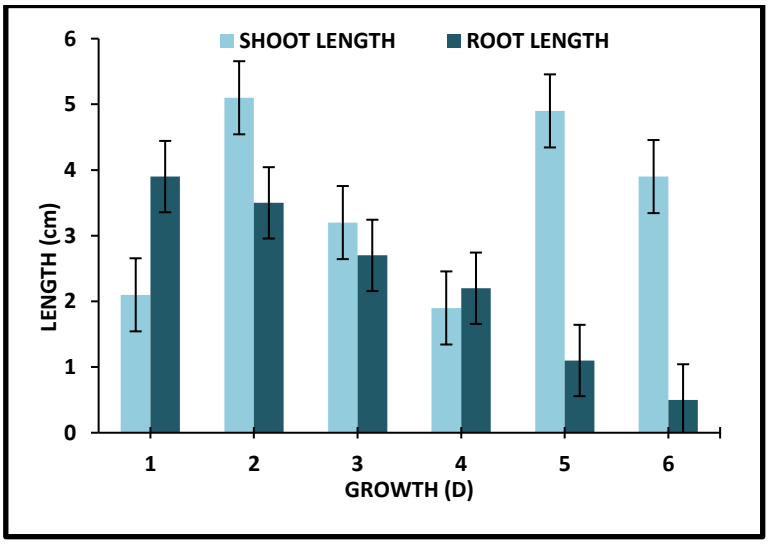

Fig. 9: Growth of Trigonella Foenum Graecum in Pot C2.

3.3. Toxicity of mixed reative azo dyes on microbes

Agar well diffusion method was used to study the toxicity of azo dyes on pathogenic bacterial strains. *Klebsiella pneumonia* was sensitive to azo dyes as the zone of inhibition towards 100 ppm was 1.3 cm and the zone of inhibition towards 200 ppm was 1.7 cm. *Staphylococuss aureus* was sensitive to 200 ppm of mixed dye. Whereas *Proteus mirabilis* and *Escherichia coli* were resistance to azo dyes as they were able to take up azo dyes as their sole carbon source. Table 2 and figure 13 Toxicity of mixed reactive azo dyes on the microbes.

Table 2: Toxicity of Mixed Reative Azo Dyes on Microbe

ORGANISM	100 ppm	200 ppm
Klebsiella pneumonia	1.3cm	1.7cm
Proteus mirabilis	-	-
Staphylococuss aureus	-	1cm
Escherichia coli	-	-

Klebsiella pneumonia ***Proteus mirabilis***

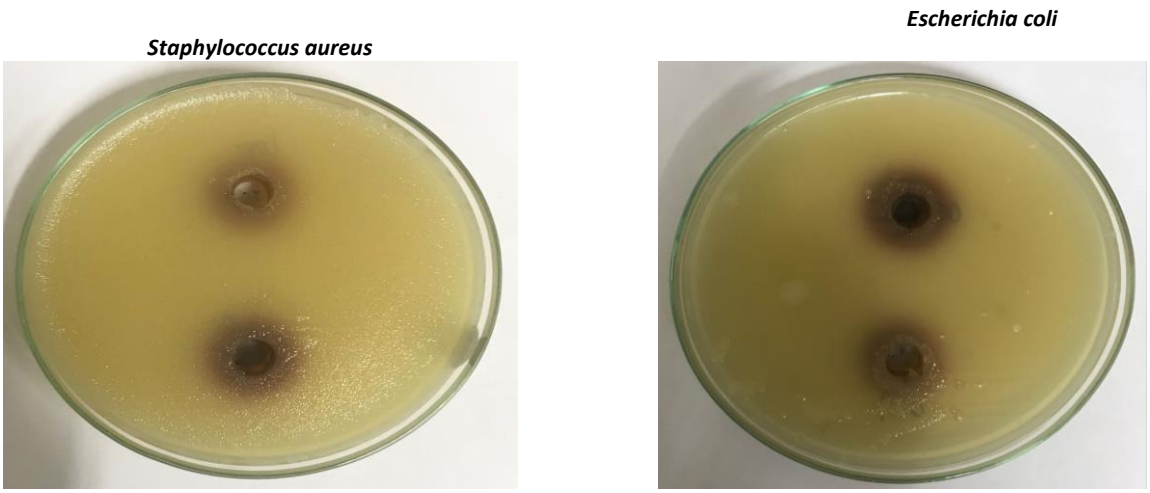

Fig. 13: Agar Plates Showing Toxicity of Mixed Reactive Azo Dyes on Microbes.

3.4. Earthworm toxicity

he protein content of the earth worm's head, body, and clitellum decreased gradually in 100ppm, 250ppm, and 500ppm while compared with that of control, which symbolizes the proteins are released from their cutaneous layer to sustain in stress condition. Figure 14 symbolizes the toxicity of azo dyes on earthworms and figure 15 is the pictorial representation of worms during protein analysis.

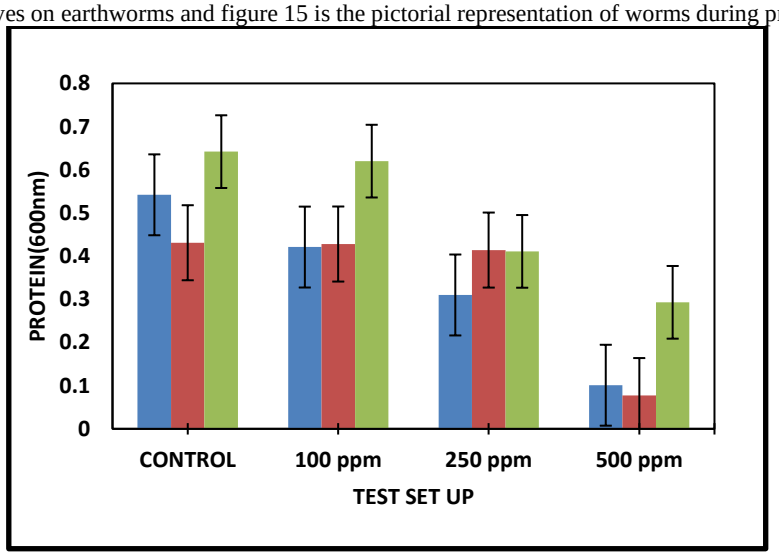

Fig. 14: Azo Dye Toxicity on Earth Worms.

Fig. 15: Earthworm's Head, Body, and Clitellum.

3.5. Phytoremediation

he percentage degraded reactive azo dye by Ceratophyllum in 20ppm showed 44.14% degradation of reactive red, 50.63% degradation of Reactive black, 44.19% of reactive brown and 49.57% degradation of mixed dyes. The percentage degraded reactive azo dye by Ceratophyllum in 50ppm showed 76.44% degradation of reactive red,84.03% degradation of Reactive black, 71.11% of reactive brown and 74.64% degradation of mixed dyes. The percentage degraded reactive azo dye by Ceratophyllum in 100ppm showed 30.83% degradation of reactive red,32.07% degradation of Reactive black, 35.2% of reactive brown and 19.41% degradation of mixed dyes. The plant died in 1000 ppm of dye sample was not able to sustain itself more than 100 ppm of mixed reactive azo dyes(figures 16-18).

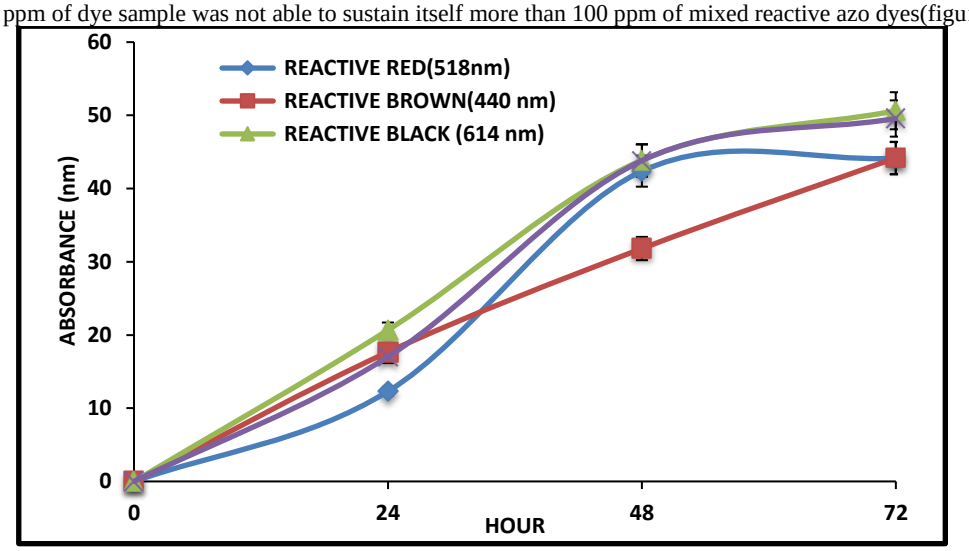

Fig. 16: Degradation of Reactive Mixed Azo Dye (20ppm) by Ceratophyllum Demersum.

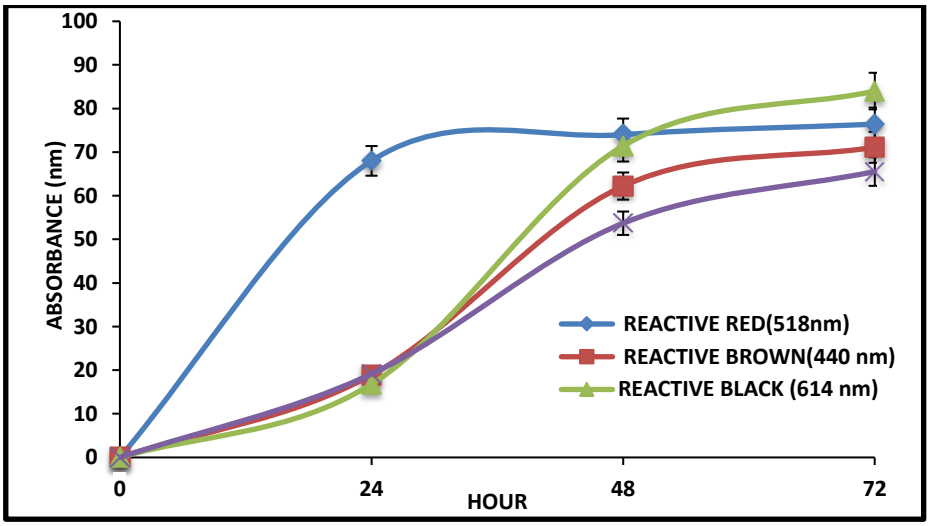

Fig. 17: Degradation of Reactive Mixed Azo Dye (50ppm) by Ceratophyllum Demersum.

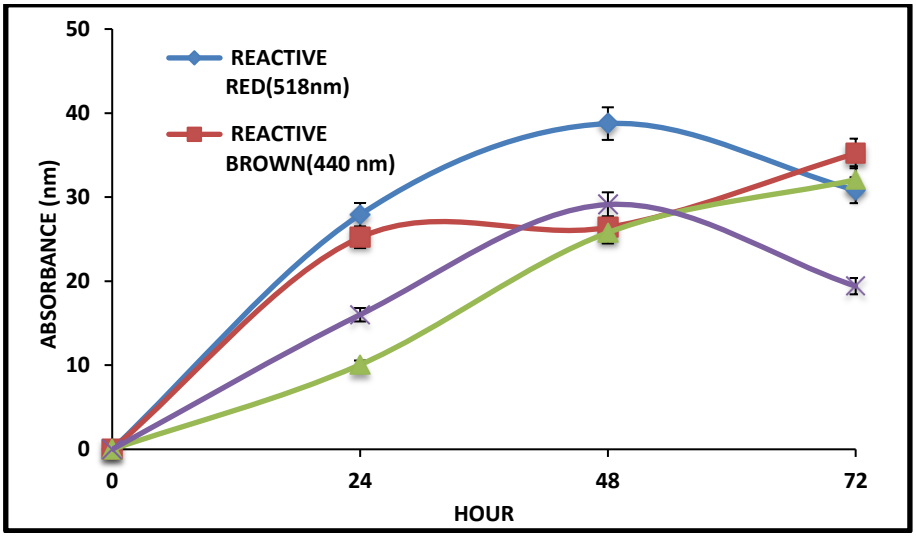

Fig. 18: Degradation of Reactive Mixed Azo Dye(100ppm) by Ceratophyllum Demersum.

3.6. Photosynthetic pigments analysis

The chlorophyll 'a' content of the test plant in 20ppm decreased to 0.0072mg/g from 0.0356mg/g. The chlorophyll 'b' content of the test plant increased to 0.3226 mg/g from 0.3025mg/g. The carotenes and xanthophylls content of the test plant decreased to 0.6383mg/g from 0.7422mg/g. The chlorophyll 'a' content of the test plant in 50ppm decreased to 0.0037mg/g from 0.0356mg/g. The chlorophyll 'b' content of the test plant increased to 0.3875 mg/g from 0.3025mg/g. The carotenes and xanthophylls content of the test plant increased to 0.8248mg/g from 0.7422mg/g. (figure 19 and table 3).

Fig. 19: Photosynthetic Pigment Extraction from Ceratophyllum Demersum.

Table 4: Photosynthetic Pigment Analysis

PIGMENT	CONTROL	20 ppm	50 ppm
CHLOROPHYLL a	0.0356mg/g	0.0072mg/g	0.0037mg/g
CHLOROPHYLL b	0.3025 mg/g	0.3226mg/g	0.38756mg/g
CAROTENES AND XANTHOPHYLLS	0.7422mg/g	0.6383mg/g	0.8248mg/g

4. Discussion

Karthikeyan and Kanchana (2014) found that the germination percentage of five different agricultural crop seeds (Paddy, Groundnut, Maize, Blackgram and Greengram) was less with untreated textile dye effluent treatment when compared to the bacterial consortium (*Bacillus odyssey, Bacillus thuringiensis, Bacillus subtilis*) treated textile dye effluent and water. The untreated textile dye effluent affected the length of plumule and radical significantly.

In the present study, the seeds of fenugreek showed stunted growth in soil contaminated by 1000 ppm of mixed dyes whereas they significantly showed a good average root and shoot length in the contaminated soils treated by free and consortium cells of Bacillus subtilis and *Enterobacter cloacae*.

Gupte et al., in 2013 on performing earthworm toxicity found that reduction in total protein content upon long-term exposure to chemical fertilizer and this decrease in protein content was due to a mechanical lipoprotein formation which may be used to repair the damages to various tissues and organs and on performing microbial toxicity found that, Different concentrations, 100– 400mgl of untreated and decolorized Red BS and 100–700mgl of Methyl Red dye solutions, were used and tested against certain microbial cultures such as *E. coli, A. vinelandii, and A. brasilence*. Zone of inhibition was obtained with untreated Red BS and Methyl red dye solutions and in contrast, no zone of inhibition was obtained with the decolorized samples as well as reference

In the present study the azo dyes of 100,250 and 500 ppm, showed toxic effect on earthworms with decrease in total protein content and left the worms sluggish and slow. About 100ppm of mixed azo dyes were toxic to *Klebsiella pneumonia and Staphylococuss* aureus Whereas Proteus mirabilis and Escherichia coli was resistance to azo dyes as they were able to take up azo dyes as their sole carbon source. Rane et al., (2015) found that the alligator weed *A. philoxeroides* had the ability to survive in extreme environmental conditions could completely de-colorize RR dye within 72 hours. Anuprita (2014) found that *Sorghum vulgare* and *Phaseolus mungo* were potent degraders of textile effluent of 70% within 12 days.

In the present study, phytoremediation was carried out *by Ceratophyllum demersum*. This has its ability to sustain itself in 20 and 50 ppm. The percentage degraded reactive azo dye by *Ceratophyllum* in 20 ppm showed 44.14% degradation of reactive red, 50.63% degradation of Reactive black, 44.19% of reactive brown and 49.57% degradation of mixed dyes. The percentage degraded reactive azo dye *by Ceratophyllu*m in 50ppm showed 76.44% degradation of reactive red, 84.03% degradation of Reactive black, 71.11% of reactive brown and 74.64% degradation of mixed dyes. The percentage degraded reactive azo dye by *Ceratophyllum* in 100ppm showed 30.83% degradation of reactive red, 32.07% degradation of Reactive black 35.2, % of reactive brown and 19.41% degradation of mixed dyes. Hence phytoremediation by *Ceratophyllum demersum* is not a wise choice in degrading mixed reactive azo dyes above 100 ppm.

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