CHAPTER 3

Materials and method

3.1 Dyes and other chemicals

Brilliant green (BG)($C_{27}H_{33}N_2.HO_4S$) and Congo red (CR) ($C_{32}H_{22}N_6O_6S_2Na_2$) dyes (**Fig. 3.1**) used in this study were of analytical grade and acquired from Sigma Aldrich, while the other chemicals utilized were purchased from Gyan Scientific in Varanasi, India. The MSM was composed of (g/L): $K_2HPO_4.2H_2O$ (1.0); KH_2PO_4 (1.0); NaCl (0.5); $MgSO_4.7H_2O$ (0.3); $(NH_4)_2SO_4$ (0.3); $CaCl_2$ (0.02); $FeCl_3$ (0.0023); $MnSO_4.H_2O$ (0.005); $ZnSO_4$ (0.005); $(NH_4)_6.Mo.7O_2$ (0.0001). The stock solution of the dye (1000 mg/L) was prepared by mixing 1.0 g of dye in 1 liter of sterilized distilled water. To prepare the synthetic wastewater, an adequate amount of stock solution was added to MSM. Congo red and brilliant green are the most used water-soluble dyes. It has been widely utilized by various textile industries as a coloring agent. The purity of all other chemicals used in this study was greater than 99.9% and they were all analytical grades.

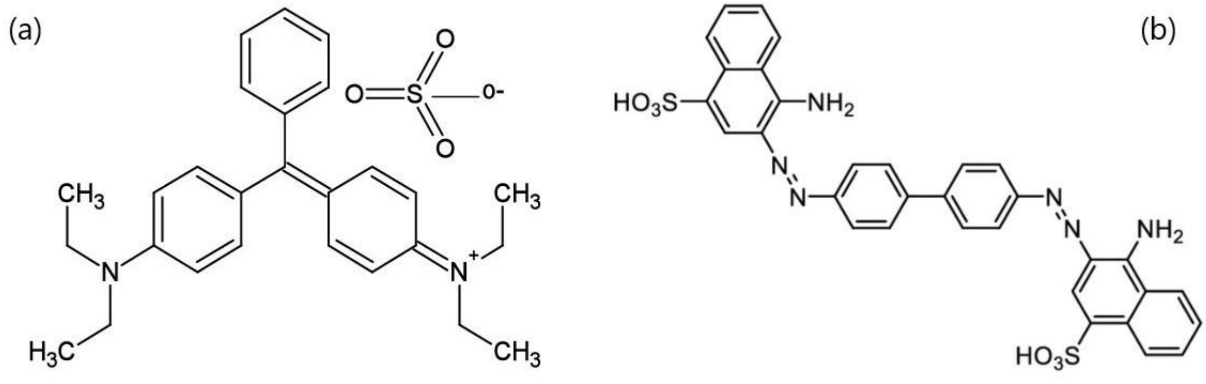

Fig. 3.1 Molecular structure of (a) Brilliant green dye (b) Congo red dye.

3.2 Isolation of effective bacterial species and identification

The dye-contaminated soil was obtained from Bhadohi, UP, India (25.3805° N, 82.5677° E) where many medium and small-scale industries are associated with the production of textiles products and carpets. The soil samples were obtained from the discharge point of the carpet industry where the soil is in contact with dye for a long time. The soil samples were supplemented with BG dye (10 mg/L) and MSM (100 mL) in a volumetric flask (250 mL) and kept in an incubator at 35℃ for 1 week. After 1 week, 4.0 mL of inoculum was transferred into another flask containing MSM (100 mL) and BG dye (40 mg/L) for further acclimatization. This process continued 4 times by increasing BG dye concentration. After that, the most suitable bacteria were isolated by serial dilution and plating methods.

The potential bacterial species was characterized at Bioraj laboratories, Nagpur, India. For the extraction of the DNA, the CTAB method was used and then purified using a DNA purification kit. The purified DNA was then amplified by PCR (polymerase chain reaction) method using the 16S-rRNA forward (8F-5´ AGAGTTTGATCCTGGCTCA3´) and reverse (1492R-5′ GGTTACCTTGTTACGACTT3´) primers. The strain was identified as Bacillus licheniformis and sequencing was then submitted to the Gen Bank database of NCBI and from there we got an accession number.

3.3 Batch study

The synthetic dye wastewater was biodegraded using a selected bacterial isolate at various initial dye concentrations ranging from 50 to 250 mg/L. 100 mL of the synthetic dyeing water with known initial dye concentration was incubated with a given bacterial isolate with known initial inoculum size at 35°C on a shaker at 120 rpm. Every 8 hours, a known amount (2 mL) sample was taken to monitor the dye concentration and bacterial mass at different time steps. Each sample was centrifuged, and the absorbance of the supernatant was measured with a UV-Vis spectrophotometer at 630 nm wavelength to determine the dye concentration.

3.4 Analytical Method

3.4.1 Decolorization, morphological, and FTIR study

UV-visible absorbance can be used to evaluate the quality of textile effluent as well as the efficacy of a treatment procedure. Visible light can be absorbed by dyes, but this ability disappears as they deteriorate. Azo dyes, in particular, are prone to azo link breakage, which results in the production of aromatic amines and the loss of color. The absorbance of the biodegraded sample was measured by UV-Vis spectrophotometer (ELICO SL 159). The % degradation was then calculated using the following equation.

$$\%\,Dye\ degradation = \left[\frac{Abs_i - Abs_o}{Abs_i}\right] * 100 \tag{3.1}$$

Where Abs_i and Abs_o refer to initial and final absorbance respectively.

SEM was used to analyze the morphology of the biofilm that formed on the LDPE surface (QUANTA 200 F, Netherlands). The immobilized carrier was vacuum-dried overnight in a 32±1.0 °C oven. For improved SEM resolution, the support material was coated with gold particles. The SEM analysis was carried out under low pressure to avoid any damage to the immobilized bacteria.

FTIR analysis of the control and biologically treated samples were carried out using KBr pellets in the wavenumber region of 400–4000 cm^{-1}. For the analysis, 15 mL of the sample was collected after biodegradation and centrifuged for 15 minutes at 8000 rpm. The supernatant was then extracted using an equal volume of ethyl acetate in a separatory funnel after being filtered through a 0.22 μm filter. The solution was then evaporated in a rotary vacuum evaporator and the remaining solution was then dissolved in HPLC-grade methanol for further analysis.

3.4.2 Metabolites identification using GC/MS

The sample (15 mL) was collected after degradation and centrifuged at 8000 rpm for 10 min and the supernatant was filtered using a 0.22 μm syringe filter. The sample was then extracted with an equal volume of methyl-tert-butyl ether (MTBE) in a reparatory funnel. Excess water was then removed using anhydrous Na_2SO_4 and the sample was pool evaporated using a rotary vacuum evaporator (with water bath temperature 45-50°C). The residue obtained after drying was then dissolved in methanol (HPLC grade) for GC/MS analysis. The GC/MS analysis was performed using Agilent GC/MS (GC 7890B, 5977B GC/MSD) equipped with HP- 5 MS column (30 m × 0.25 mm × 0.25 micron) using helium as carrier gas. The injector temperature was maintained between 250 – 300°C. The metabolites were identified by retention time and corresponding mass spectra.

3.5 Toxicity Assessment

Vigna radiata seeds were utilized for phytotoxicity analysis. The healthy seeds, which were uniform in size and color, were surface sterilized. After being thoroughly cleaned with distilled water initially, the seeds were sterilized for about 2 minutes with 1% (v/v) sodium hypochlorite and allowed to dry at room temperature. The effects of dye treatment on the activities of antioxidant enzyme superoxide dismutase (SOD) and photosynthetic plant pigments were assayed.

3.5.1 Phytotoxicity

The phytotoxicity of BG dye was assessed by seed germination test using *Vigna radiata* seeds. The biologically treated sample was collected and centrifuged at 6000 rpm for 15 min and supernatant was filtered using filter paper for further analysis. Firstly, the seeds of uniform size were sterilized using a 5% sodium hypochlorite solution and then washed thoroughly with distilled water. The treated and untreated dye water samples were then used to irrigate seeds that had been placed in a petri dish on filter paper. In a separate petri-plate, *Vigna radiata* seeds were irrigated with distilled water and kept as a control. These petri-plates were then incubated in a BOD incubator (NSW-256) for 7 days at 30°C (Kishor et al., 2021b). After two days seed germination was observed and seeds were taken out after 7 days for the assessment of their germination, root, and shoot length. All the experiments were performed in triplicates.

3.5.2 Photosynthetic pigment content

The Lichtenthaler (1987) technique was used to assess the chlorophyll a, b and carotenoid content of fresh leaves. Plant leaves samples were grounded in mortar and pestle and extracted with 80% acetone. For the analysis of chlorophyll a, b, and carotenoids, the optical density of the extracted sample was evaluated at various wavelengths of 662 nm, 645 nm, and 470 nm, respectively.

3.5.3 Bacterial Toxicity

Bioluminescent bacteria such as *Aliivibrio fischeri*, *Vibrio qinghaiensis*, *Photobacterium phosphorum* have been used by several researchers for the toxicity assessment (Mirjani et al., 2021; Yang et al., 2022). Bioluminescence nature of bacterial species are used for toxicity assessment because of their property of light emission via Luciferase- catalyzed reaction, which involves reduction of Flavin mononucleotide (FMN) to generate coenzyme $FMNH_2$. The educed $FMNH_2$ and long-chain aldehydes were further catalyzed into FMN and fatty acid by following equation (Yang et al., 2022).

$$\mathrm{FMNH_2 + RCHO + }O_2 \longrightarrow \mathrm{FMN + RCOOH + }H_2\mathrm{O} + h\nu$$

In the present study *Photorhabdus luminescence subsp. Akhurstii* (MCC 3251) was used as bioluminescent bacteria. Untreated and biologically treated dye samples were tested for acute and chronic toxicity. Firstly, the bacterial slant obtained from NCMR was cultured into LB medium (Luria Bertani) and kept it at 25℃ for 24 h. The culture was taken when it reached to $\mathrm{OD}_{600} = 1.5$ value. Then 1 mL of *Photorhabdus luminescence subsp. Akhurstii* (MCC 3251) was inoculated into 5 mL of each sample (i.e., untreated dye sample (UT), biologically treated dye sample (BT), and distilled water sample as control (DW)) for further bioluminescence analysis. The % inhibition was calculated using:

$$I = \left(1 - \frac{L_s}{L_c}\right) \times 100 \qquad (3.2)$$

where I is the inhibition ratio and L_s and L_c is the intensity of sample and control respectively.

3.5.4 Superoxide dismutase (SOD) enzyme activity assay

Superoxide dismutase (SOD) activity was measured by inhibition in nitrobluetetrazolium (NBT) photo-chemical reduction caused by the SOD enzyme. The SOD activity was determined using the method of Winterbourn (1976), which is based on the principle that Superoxide radicals photo reduce NBT, resulting in the formation of blue-colored formazan. The crude extract was prepared by homogenizing root and shoot grown individually in distilled water (DW), Untreated (UT), and Biologically treated (BT) in 2 mL of extraction buffer containing 50 mM phosphate buffer (pH 7.5), 0.1 mM EDTA, 2% (w/v) polyvinylpyrolidone (PVP). The sample was centrifuged at 12000 rpm for 10 min at 4℃. The supernatant was collected and 100 µL sample was mixed with 12 mM L-methionine, 50 µM NBT, and 10 µM riboflavin to obtain the final reaction mixture. This mixture was then incubated at 35℃ for 20 min under exposure to light. The absorbance was measured at 560 nm. SOD activity was measured in units (U), with one unit (U) equaling the quantity of enzyme exhibiting a 50 % inhibition of photochemical degradation of NBT.