3 Material and Methods

3.1 Materials and Chemicals

The analytical grade Congo Red dye (λ_{max}: 496.5 nm), nutrient agar (consisting of [in g/L] peptic digest of animal tissue 5.0, beef extract 1.5, yeast extract 1.5, sodium chloride 5.0, agar-agar 15.0) and nutrient broth (consisting of [in g/L] peptone 5.0, sodium chloride 5.0, meat extract 1.5, yeast extract 1.5) were purchased from Merck (Darmstadt, Germany). Congo Red is one of the most popular water-soluble azo dyes. It has been widely used by the textile industry as a synthetic colorant due to its properties such as a large variety of shades and resistance to decoloring. All the other chemicals used in this study were of analytical grade with more than 99.9 % purity.

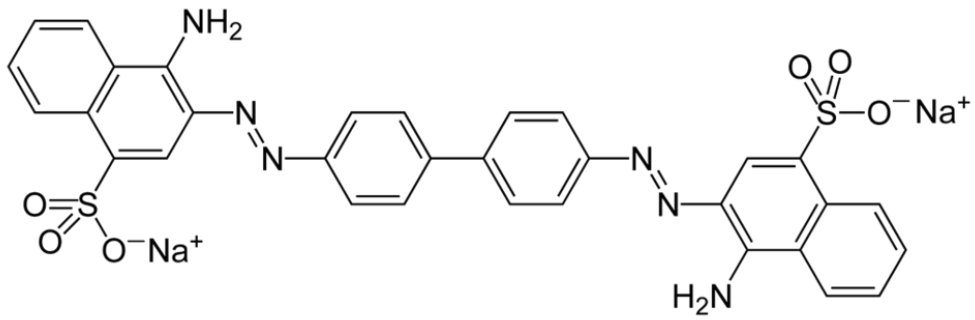

Figure 3.1 Molecular structure of Congo Red dye

Shake flasks, beakers, measuring cylinders, glass bottles, test tubes, volumetric flasks, petri-dishes, culture tubes, L-shape glass rod, conical flasks, flat bottom flasks, desiccators, round bottom flasks, reagent bottles, etc. were used which were made up of boro-silicate glass. Before use, all the glass wares were sterilized by hydrochloric acid and rinsed adequately with distilled water. The materials like sterilized polybag, parafilm, micro-pipette and micro-pipette tube, Eppendorf tubes, silicon tube, cotton bundles, inoculation loop, were purchased from Borosil®, India.

3.2 Preparation of synthetic dye wastewater

As the actual wastewater is the mix of multiple dyes and their individual compositions are often unknown; therefore, we used the simulated (synthetic) dyeing water to study the bacterial growth, mass transfer and dye utilization kinetics. To generate synthetic textile wastewater of varying concentrations (50-800 mg/L) to be employed in this investigation, the Congo Red dye was added to the MSM (mineral salts medium) at the necessary amount. The composition of MSM used here is given in g/L as follows: K_2HPO_4 (1.6); KH_2PO_4 (0.4); $MgSO_4.7H_2O$ (0.2); NaCl (0.1) and $CaCl_2$ (0.02) with 1.0 mL/L of trace element stock solution and 1mL of $FeSO_4.6H_2O$ stock solution of concentration 5g/L. The composition of trace element solution was taken in g/L as follows: $MnSO_4.H_2O$ (1.8); $ZnSO_4$ (0.2); $CuSO_4$ (0.1); Na_2MoO_4 (0.25) and H_3BO_3 (2) (Geed et al. 2017). The media was autoclaved for 20 minutes at 121° C. In addition, 10 mL of a 1 percent glucose solution was supplied as a co-substrate in the RFBB influent after every 7 days of operation to stimulate bacterial growth. The composition of actual wastewater is slightly different from the simulated dyeing water that we have used in our studies. Enrichment and isolation of bacterial species

The bacterial species were obtained from the soil samples collected from a pond (25.3805° N, 82.5677° E) near a dyeing-waste discharge site of a carpet-dyeing unit at Bhadohi in U.P., India. The NaCl-yeast (NY) extract-based medium consisting of 4% NaCl and 0.5% yeast was used to grow the bacterial species present in the soil sample (Abu Talha et al. 2018). A 5 g soil sample was added in a 100 ml NY medium and incubated for 72 hours at 30 deg C. A serial dilution of the suspension was carried out, and 10 different isolates (labeled as A to J) were prepared. These isolates were further purified by making their sub-cultures twice, and their liquid cultures were prepared in nutrient broth.

3.3 Molecular characterization of the efficient isolated bacteria

The molecular characterization of the isolated species was done at the Microbial Type Culture Collection (MTCC) lab (Chandigarh, India). Genomic DNA was isolated from the

pure culture using the Bacterial DNA MiniPrep kit (Make: Zymo Research). 16S rRNA gene was amplified through PCR using universal 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The PCR product was visualized on 1% Agarose gel. The PCR amplicon was gel eluted and purified using QIA quick Gel Extraction Kit (Make: Qiagen). A purified PCR product was sequenced using the Sanger DNA sequencing method, and the obtained sequences were visualized and analyzed using Finch TV software ver 1.4. The assembled nucleotide sequences of the 16S rRNA gene were subjected to a similarity search in the NCBI (National Centre for Biotechnology Information) database using BLAST tool. Precisely, the nucleotide sequence was aligned with published sequences from the NCBI database using the multiple sequence alignment program of MEGA X 10.1 (Molecular Evolutionary Genetics Analysis). The neighbor-joining algorithm of MEGA X 10.1 was used to generate the phylogenetic tree (Kumar et al. 2018). The homologous DNA sequence was initially identified and acquired using the Basic Local Alignment Search Tool (BLAST). The next step was the sequence alignment, which included arranging the sequences to determine the similarity regions. These similarities may be due to some evolutionary, structural, and functional relationships among the sequences. Finally, the phylogenetic tree was constructed by choosing the neighbor-joining method.

3.4 Biodegradation experiments in a batch reactor

Biodegradation of the synthetic dye wastewater was carried out at different initial dye concentrations ranging from 50 to 250 mg/L using a selected bacterial isolate. 100 mL of the prepared synthetic dyeing water of Congo Red with known initial dye concentration was incubated with a given bacterial isolate of known initial inoculum size at 35 °C on a shaker at 120 rpm. A known amount (2 mL) sample was taken out every 8 hours to monitor the dye concentration and bacterial mass at different time steps. Each sample was centrifuged, and the absorbance of the supernatant was measured using a UV-Vis spectrophotometer to determine the dye concentration [$S(t)$]. Majority of the consumed mass is wasted in the form of by-

products produced during the bacterial metabolism. Since there was negligible adsorption of dye to the biomass (which was observed by a light pinkish color of biomass), the adsorption of dye to the bacterial mass was not taken into consideration. Next, the settled bacterial mass in the centrifuged sample was filtered out using a 0.22 µm filter paper assembly and re-suspended in the double-distilled water. This process was repeated five times to remove all the residual dye, nutrients, and metabolites. The final filtered bacterial mass was dried at 60 °C for 12 hours and re-suspended in the double-distilled water of the same volume as the initial sample size (i.e., 2 mL) to make the final suspension of the bacterial mass. The optical density of the final suspension was measured and, after that, the bacterial cell mass-concentration [$X(t)$] was estimated using the calibration plot. This calibration plot consists of the corresponding dry cell mass (gm). All the experiments were done in triplicates to determine deviations in the measurements.

3.5 Bioreactor configuration and immobilization of packing material

A cylindrical borosilicate glass (internal diameter = 6 cm; height = 53 cm) based bioreactor was fabricated with a total volume of around 1500 mL and a working volume of 1200 mL for biodegradation SDW. A total of 2000 mL of SDW was processed in each run with varying dye concentrations. Ports were provided at the top for sample collection and bottom for air supply, influent feeding, and drainage. For the immobilization process, washed and dried LDPE foam cubes were filled inside the reactor up to a height of 43 cm. The fixed bed of packing material was prepared using stainless steel sieves provided at the top and bottom of the packing. LDPE (low-density poly-ethylene) foam purchased from the local market was cut into approximately 1 cm3 size cubes. Foam pieces were washed thoroughly with distilled water, followed by the absolute ethanol thrice and finally with the distilled water. They were squeezed and dried overnight in an oven at 60 °C. Next, these dried foam pieces were used as a packing medium in the AnA-RFBB. Batch experiments of Congo red

dye adsorption were studied on the LDPE foam as adsorbent. Results indicated that LDPE foam had negligible adsorption capacity for Congo red dye.

3.6 Analytical methods

Textile effluent is challenging to analyze due to its complexity. However, knowing the effluent's composition is critical for selecting a suitable treatment approach. The effluents must be analyzed to assess the treatment's efficacy. If present, the elimination of nutrients and organic content should be measured, and the breakdown of dyes and other hazardous or recalcitrant organic compounds. The dye preparations must be analyzed since they include active dye molecules and by-products. Toxicity should also be monitored to ensure that the treated effluent does not affect the receiving water body or its ecosystems. The samples were collected and analyzed for color, biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total organic carbon (TOC). Before analysis, the samples were centrifuged at 8000 rpm for 10 min and filtered through 0.22 µm filters.

3.6.1 UV-Vis Spectrophotometry

The capacity of dyes to absorb light is a simple but accurate approach for evaluating their deterioration. UV-visible absorbance may be used to assess the quality of textile effluent and the efficiency of a treatment procedure. Dyes can absorb visible light, but they lose this property when they deteriorate. Azo dyes, in particular, are prone to breakage of the azo link, resulting in the production of aromatic amines and the loss of color. Although colorless, these amines are poisonous and mutagenic and must be degraded further, potentially into inorganic chemicals. The absorbance of the treated dyeing water was monitored using a UV-Vis spectrophotometer (Model No.: SL-159, Elico, India) at 497 nm to assess the decolorization.

3.6.2 Biochemical Oxygen Demand (BOD)

The amount of oxygen required for the biological oxidation of organic materials in the effluent is BOD. It is determined by measuring the difference in dissolved oxygen over five days in a sample. The initial DO content of a given sample volume is recorded, and the

sample is removed from the incubator after a five-day incubation period at 20°C to get the final DO content. A higher BOD means more oxygen is required, implying that the water quality is poor. Low BOD indicates that less oxygen is taken from the water, suggesting that the water is typically purer.

3.6.3 Chemical Oxygen Demand (COD)

The COD measures how much oxygen is required to chemically oxidize the organic and inorganic components in the effluent. COD is calculated because, given acidic conditions, a powerful oxidising agent may completely oxidise almost all organic ingredients to carbon dioxide. Under acidic conditions, COD is frequently measured using a strong oxidant (e.g., potassium dichromate or potassium permanganate). The oxidant is introduced to the sample in a concentration that is known to be in excess. After the oxidation process is complete, the amount of oxidant left in the solution is measured to determine the concentration of organics in the sample. Titration with an indicator solution is commonly used to accomplish this. The mass of oxygen used per liter of solution is measured in milligrams per liter of COD. The sample oxidation was done using potassium dichromate in a digestor (Uniphos COD digestor, Gujarat, India). The absorbance is measured for the Cr^{3+} ions generated in the Uniphos COD analyzer.

3.6.4 Total Organic Carbon (TOC)

The TOC determines the total amount of organic carbon in an effluent, including that which cannot be oxidized. The concentration of additional nutrients, such as inorganic ions, should be tested in addition to the organic content. Salts, particularly sodium chloride, which are employed in high quantities to enhance dye binding to fabric and are consequently prevalent in the resultant effluent, should be given special attention. The TOC analysis was done by performing combustion of the sample at 950 ºC in a TOC analyzer (Model No.: multi N/C 2100, Analytic Jena, Germany).

3.8 Toxicity Analysis

Although several chemical-biological processes have been demonstrated to have effective remediation potential for the dyeing wastewater, the toxicity level in the treated solution remains a concern (Bilal et al. 2016; Bilal and Asgher 2015; Iqbal and Bhatti 2015). The samples of the treated dyeing water were collected, centrifuged at 8000 rpm for 10 min., and filtered through a 0.22 µm filter paper. Next, the supernatant parts were separated and sterilized for the following phytotoxicity and bacterial-toxicity analyses.

3.8.1 Phytotoxicity

The phytotoxicity assessment was carried out with the seeds of *Vigna radiata* for the SDW and corresponding treated dyeing water samples. The *Vigna radiata* seed was chosen due to its easy availability in Northern India and frequent usage as a test plant by the researchers for toxicity assessments in the literature (Kannan and Upreti 2008; Bhattacharya et al. 2020). The systematic methodology was based on the guidelines described elsewhere (Bharagava et al. 2018; Saratale et al. 2019). The seeds were sterilized with 5 % sodium hypochlorite solution for 10 min. and washed thoroughly to ensure complete removal of sodium hypochlorite. These seeds were then immersed in the untreated- and treated-dyeing water samples. The *Vigna radiata* seeds were also immersed in the distilled water (DiW) separately as control. After 5 days, the seeds were taken out and assessed for their germination, root, and shoot lengths. All the experiments were carried out in triplicates, and the average data were reported in the presented study with the standard error.

3.8.2 Bacterial toxicity

Bioluminescent bacteria such as *Vibrio fischeri*, *Pseudomonas fluorescens*, and *Vibrio harvey* bacterial assays have been conventionally employed for toxicity analysis (Girotti et al. 2008; Gatidou, Stasinakis, and Iatrou 2015). The basic biochemical mechanism of bioluminescence involves the reduction of flavin mononucleotide (FMN) to reduced flavin mononucleotide ($FMNH_2$) *via* the action of the luciferase enzyme (Meighen 1993). This

reduced flavin mononucleotide and a long-chain aliphatic aldehyde are further oxidized by molecular oxygen as shown in the following reaction (Dunlap 2014):

$$FMNH_2 + R\text{-}CHO + O_2 \longrightarrow FMN + R\text{-}COOH + H_2O + light \qquad (3.1)$$

This study used *P. luminescens subsp akhurstii* as a bioluminescence assay to assess the acute and chronic toxicities of the treated SDW samples. The luminescent bacteria were obtained from the National Centre for Microbial Resource (Pune, India). The bacterial slant obtained was further cultured in LB broth (Luria Bertani broth) for further carrying out the bioluminescence toxicity analysis. The bacterial culture with the optical density of unity was used with the samples for toxicity measurement. 1000 µL of *P. luminescens subsp akhurstii* was added to 5000 µL of each sample, e. g. distilled water as control (DiW), SDW, anaerobically treated dyeing water (AnTDW), and anaerobically-aerobically treated dyeing water (AnATDW) for all bioluminescence measurement assays. The term % inhibition was defined as

$$\%\, Inhibition = \left[1 - \frac{sample\ intensity}{control\ intensity}\right] \times 100 \qquad (3.2)$$

The bioluminescence intensities (in counts per second) of the treated, untreated, and control water samples were obtained using *Horiba Fluorescence spectrophotometer (Model No.: PTI QuantaMaster™ 8000 series)*. The acute and chronic toxicities were measured after 30 min and 24 h, respectively, after adding *P. luminescens* to different samples.