Chapter 4

Biodegradation of Congo red dye using *Lysinibacillus* species in a moving bed biofilm reactor: Continuous study and kinetic evaluation

4.1. Introduction

Synthetic dyes have various applications in the textile, slaughterhouse foods, beverages, and paper printing industries. Based on the chemical bonding of chromophores, synthetic dyes are categorized into five categories: nitro, azo, indigo, anthraquinone, and triarylmethane. Among these, azo dyes are extensively produced and used for dyeing purposes since they exhibit excellent antimicrobial and high stability characteristics (Sharma et al., 2021; Mazeau and Wyszomirski, 2012). Globally more than 7×10^5 metric tons of azo dyes have been produced annually, and 5–10 % of dyes are released into aquatic bodies (Hakimelahi et al., 2012; Yao et al., 2016). In specific, Congo red (CR) dye is a benzidine-based anionic azo dye used in the textile, leather, pharmaceuticals, food processing, and paints industries (Sharma et al., 2021). The sulphonate group (SO_3^-) in CR dye facilitates the electrostatic interaction with the textile fibres and enables a wide range of coloration (Sharma et al., 2021; Mazeau and Wyszomirski, 2012). The wastewater released from these industries contains colored pigments, adversely affecting the aquatic system by decreasing visibility, sunlight penetration, and oxygen diffusivity (Sinha et al., 2016; Taheri et al., 2014; Giovanella et al.,2020). Moreover, it has become a primary concern to environmentalists due to its mutagenicity, carcinogenicity, and xenobiotic nature (Calderón et al., 2012; Pepio et al., 2011: Zhang et al., 2005).

In the recent past, several physicochemical (filtration, coagulation, membrane separation, adsorption, etc.), advanced oxidation (ozonation, UV/H_2O_2, etc.), and biological processes have been used for the treatment of dye-containing wastewater (Shabbir et al., 2017; Swain et al.,2020;

Kureel et al.,2017; Modak et al.,2016; Gupta et al.,2013). Despite high degradation efficiency within a limited period by physicochemical and advanced oxidation processes, the cost and environmental factors are the major concern for these processes (Vikrant et al.,2018; Nasseri et al., 2014; Ghaly et al.,2014; Kureel et al.,2016). However, the advantages of the biological process (i.e., eco-friendliness, cost-effectiveness, and toxic byproducts elimination) help to overcome the limitation of the conventional approaches in the degradation of the azo dyes (Azizi et al., 2015a; Li et al., 2015; Li et al.,2018a).

Previously, the various bacterial species (i.e., *Providencia stuartii*, *Bacillus sp.*, *Brevibacillus parabrevis*, *Bacillus subtilis*, *Bacillus cohnii RAPT1*, and *Aspergillus niger*) have been investigated for the biodegradation of azo dye (Goswami et al.,2020; Sonwani et al.,2020; Abu Talha et al.,2018; Shalini and Setty 2019; *Padmanaban et al.,2016;* Mahmoud et al.,2017). These bacterial species are generally employed as suspended and attached (immobilized) cultures. The immobilized microbial cell is highly preferable for the biodegradation of persistent organic pollutants. The microorganisms immobilized onto the solid packing support (biocarrier) have higher degradation ability and resilient properties against adverse process conditions (Borkar et al., 2013; Swain et al.,2021; Sonwani et al.,2020). The real-time application of the immobilized biocarrier has been evaluated in various attached-growth bioreactors such as packed bed bioreactor (PBBR), moving bed biofilm reactor (MBBR), rotating biological contactor (RBC), etc. for the biodegradation of azo dye (Maurya et al., 2021; Basak et al., 2019; Cheng et al.,2019; Neetha et al.,2018). Among these, the moving bed biofilm reactor (MBBR) is a highly preferred technology for the biodegradation of toxic pollutants due to its moving carrier configuration (Sandip and Kalyanraman, 2019; Sonwani et al. (2019b)). The movement of the carriers inside the bioreactor offers better mass diffusion of substrate and high biomass development (Swain et al., .2020). The physical and chemical characteristics of the biocarriers used in the MBBR play a vital role in the biodegradation process. A low-cost and high specific surface area biocarrier is a superior choice for the immobilization of microorganisms (Sonwani et al.,2020). In this direction, polyurethane foam (PUF) is considered a suitable solid matrix due to its stable mechanical strength and high porosity (Swain et al.,2021). Similarly, polypropylene (PP), an inert, non-biodegradable, and hydrophobic plastic carrier, is used in the MBBR to treat wastewater (Kim et al., 2015). However, very few studies were reported on the application of composite biocarrier (combination of PP and PUF) in an MBBR to treat organic pollutants (i.e., CR dye).

This study aims to develop a biocarrier composed of PUF and PP and use in a lab-scale MBBR for the biodegradation of CR dye. The potential bacterial consortium (*Lysinibacillus fusiformis* KLM1 and *Lysinibacillus macrolides* KLM2) was isolated from a contaminated dye site and immobilized onto polypropylene-polyurethane foam (PP-PUF). The MBBR packed with immobilized biocarrier was operated continuously at various CR dye concentrations. Further, the substrate utilization kinetics was evaluated using the First-order and Second-order models.

4.2. Materials and methods

4.2.1. Chemicals and composition of mineral salt media

The Congo red (CR) dye (CAS number 573-58-0) was purchased from Sigma Aldrich, India. The MSM (minimal salt medium) and other chemicals have been brought from Merck, India. The composition of the MSM (g/L) was followed as; KH_2PO_4 (2.2), NaH_2PO_4 (3.6), $FeCl_3$ (0.015), $MnSO_4 \cdot H_2O$ (0.5), $MgSO_4$ (0.5), and peptone (2.0). The synthetic wastewater was prepared by adding the desired amount of CR dye in MSM containing glucose of 0.2% (w/v).

4.2.2. Enrichment of dye degrading bacterial species and isolation process

The soil sample was collected from the textile industries at Bhadohi, Utter Pradesh, (India). This site was chosen to isolate potential species because of the high probability of dye degrading microorganisms present at that location. The soil sample was collected in a sterile container. It was kept in the refrigerator at 4°C for further use. The microorganisms were enriched by adding a 5.0 g of soil sample in a 250 mL flask containing 100 mL MSM and CR dye of 50 mg/L. The flask was incubated at 36 ± 2.0 °C and 120 rpm for six days. After the incubation period, a 20 mL of sample was transferred to another conical flask with fresh 100 mL MSM containing CR dye (100 mg/L) and incubated under similar conditions. Further, the acclimatization procedure was repeated thrice by gradually increasing the CR dye concentration in each step. Finally, the potential bacterial species were isolated and stored at 4.0 °C for immobilization.

4.2.3. Bioreactor set-up and bio carrier configuration

A rectangular MBBR tank was fabricated with polyvinyl sheets with a total volume of 40 L (a working capacity of 30 L). The dimensional specifications of the MBBR and the biocarrier are provided in Table 4.1. The reactor was connected with a peristaltic pump (Milton Roy India, Chennai Model A-11 SS), compressor (XP-AC-24 L Xtra Power), rotameter (Eureka Industrial Pvt. Ltd., India), and an effluent tank (Figure 4.1). A feed tank of 200 L was used to feed the synthetic wastewater to the MBBR. The bio carrier used in the present study was a composite of polyurethane foam (PUF) and polypropylene (PP). The PUF has been purchased from a local shop near IIT BHU, Varanasi, India. The PP has been brought from Biotech, New Delhi, India. The specific area and density of PP were 550 m^2/m^3 and 930 kg/m^3, respectively. The PUF was cut into the cubic shape of dimension 2.5 ± 0.3 cm. These PUF cubes were inserted in the alternate holes of PP carriers and the composite (PP-PUF) biocarrier used in the MBBR. The isolated bacterial cultures were added as inoculum in the MBBR bioreactor to degrade the Congo red dye.

Table 4.1. Dimensional details of the MBBR and biocarrier.

Variables	**Units**	**Values**
Reactor Material type	-	Polyvinyl sheet
Length of the bioreactor	cm	38
Width of the bioreactor	cm	38
Height of the bioreactor	cm	28
Total volume of the bioreactor	L	40
Working volume of the bioreactor	L	30
Packing material type	-	Polypropylene-polyurethane foam
Filling fraction	%	40
Density of the biocarrier	kg/m^3	930
Specific surface area of the biocarrier	m^2/m^3	550
pH of the media	-	7.0 ± 0.5
Temperature	$^{\circ}C$	32 ± 2
Dissolved oxygen	mg/L	4.0 ± 0.3
Initial concentration of CR dye	mg/L	50-250

The wastewater was fed to the bioreactor with the help of a peristaltic pump (Milton Roy India, Chennai Model A-11 SS). Initially, the microorganisms were immobilized onto the PP-PUF by operating the MBBR in a batch mode with synthetic wastewater containing CR dye of 50 mg/L. The biofilm development was analyzed in a Scanning electron microscope (SEM) after 15 days of operation. After the biofilm attachment confirmation, the MBBR was switched to the continuous mode by varying the initial dye concentration from 50 to 250 mg/L at a specific flow rate. The potency of the MBBR was investigated by determining the following parameters (Sonwani et al., 2020; Geed et al., 2017).

Removal efficiency (RE, %) = $\frac{D_0 - D_t}{D_0} x100$ (4.1)

Inlet loading rate (ILR, mg/L. d) = $\frac{QxD_0}{V}$ (4.2)

Elimination capacity (EC, mg/L. d) = $\frac{D_0 - D_t XQ}{V}$ (4.3)

where D_0 and D_t are the inlet and outlet dye concentrations (mg/L), respectively. In addition, Q represents the volumetric flow rate (L/h), and V denotes the working volume of the MBBR (L).

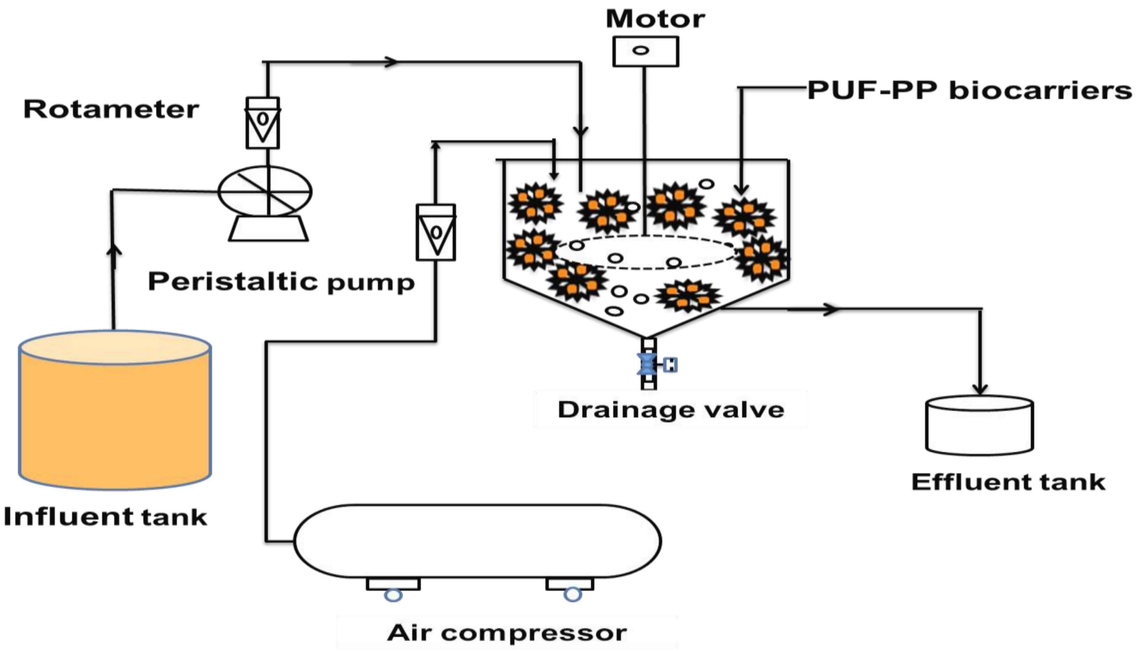

Figure. 4.1. Experimental set-up of MBBR for CR dye biodegradation.

4.2.4. Analytical methods

The UV–visible spectrophotometer (ELICO-SL 210) was used to measure the absorbance of the treated and untreated wastewater samples. Before analysis, the sample was centrifuged by a centrifuge machine (Remi R-8c Bl/R-8m) at 5000 rpm. The pH and dissolved oxygen (DO) were measured by pH meter (HD 2305.0; Delta OHM; Italy) and DO meter (HD 2109.1; Delta OHM; Italy), respectively. The morphology of the PP-PUF biocarrier was analyzed using a Scanning electron microscope (QUANTA 200F, Netherlands). All the samples were analyzed in triplicates, and the mean values were reported in the manuscript.

4.2.5. Kinetics study

The kinetic study is an analytical approach to evaluate the substrate consumption rate, which shows the effectiveness of the bioreactors for the mineralization of organic pollutants (Debik and Coskun,2009). In addition, the parameters obtained from the kinetic analysis can be used to design the process conditions to get the desired responses. This work used the First-order and Second-order (Grau) models to evaluate the substrate degradation rate.

First-order kinetic model

The CR dye biodegradation in the MBBR is assumed to follow the First-order kinetic equation as

$$D_t = D_0 e^{-Kt} \tag{4.4}$$

$$\frac{D_t}{D_o} = e^{-kt} \tag{4.5}$$

where D_o and D_t represent the initial and final CR dye concentration (mg/L), respectively. Moreover, t represents the time period (h), and k is the first-order rate constant (h^{-1}).

Now, taking the log of both sides of Eq. (4.5), we get the final expression for the First-order kinetic as

$$\text{Ln}\left(\frac{D_t}{D_0}\right) = -k.\, t \tag{4.6}$$

Second-order kinetic model (Grau model)

The expression for the Second-order kinetic can be written as

$$\frac{1}{D_t} = \frac{1}{D_0} + k_1 t \tag{4.7}$$

Now, Eq. (4.8) can be obtained by rearranging Eq. (4.7) as

$$\left(\frac{1}{D_t} - \frac{1}{D_0}\right) = k_1.\, t \tag{4.8}$$

where k_1 represents the Second-order rate constant (L/mg. h).

4.2.6. Statistical analysis

The significance of the kinetic model was verified by evaluating the root mean square error (RMSE) at each condition. The RMSE is calculated based on the following expression as follows:

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(\,Predicted\ data_i - Experimental\ data_i\,)^2}{n}} \tag{4.9}$$

where n refers to the number of data.

4.3. Results and Discussion

4.3.1. Identification of dye degrading bacterial species and characterization of biocarrier

The microbial samples were sent to Triyat Scientific, Nagpur, India, to identify the species. The DNA was extracted using the EX pure Microbial DNA isolation kit (Bogar Bio Bee stores Pvt Ltd., India), and the detailed procedure was described elsewhere (Kureel et al., 2017). In the next stage, the genomic DNA was amplified to make multiple copies of the specimen using the polymerase chain reaction (PCR) process. The PCR was run under the different thermal cycles followed by primary denaturation at 95 °C for 30 sec, annealing at 50 ºC for 30 sec, and extension at 72 ºC for 10 min. The sequencing of the PCR sample was done by the ABI 3730xl (Applied Biosystems) sequencer. The DNA sequences obtained from 16s rRNA were sent to the NCBI (Gen Bank) database (https://www.ncbi.nlm.nih.gov) to construct a phylogenetic tree of the bacterial species. These species were identified as *Lysinibacillus macrolides* KLM2 (MW599204) and *Lysinibacillus fusiformis* KLM1 (MW599200). The phylogenetic tree was constructed using MEGA 6.0 software with NJ (neighbor-joining) methods (Fig. 4.2).

Fig. 4.3 shows the images of the surface structure of the PP-PUF biocarrier before and after immobilization. It was observed that the presence of a rough surface and several micropores on the PP-PUF biocarrier helps the bacterial adhesion on the solid surface (Fig. 4.3a). The biofilm layer attachment on the surface of the biocarrier was represented in Fig. 4.3b. The porous structure of the PUF may provide a high specific surface area for the growth of bacterial cells. The CR dye consumption rate depends upon the active biomass, which releases the extracellular polymeric substance. The amount of biomass attached to the PP-PUF biocarrier was evaluated during the immobilization period, and it was found to be 967 mg/L.

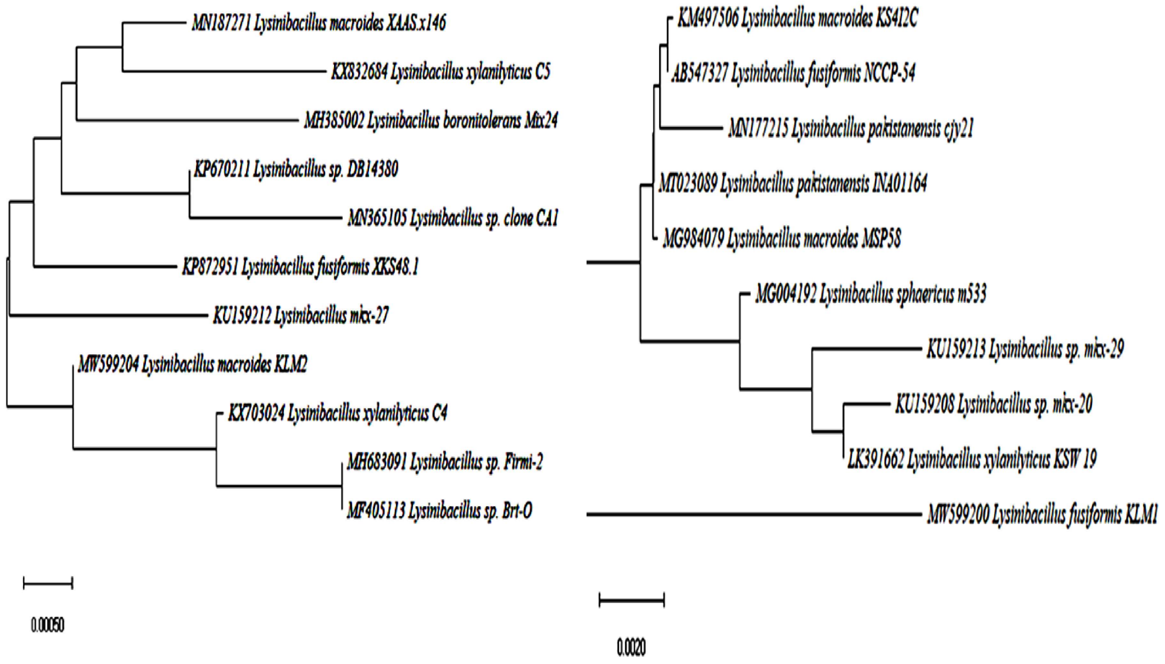

Figure. 4.2. Phylogenetic tree of isolated bacterial species *Lysinibacillus macrolides* KLM2 (MW599204) and *Lysinibacillus fusiformis* KLM1(MW599200) and by using the Neighbour-Joining method.

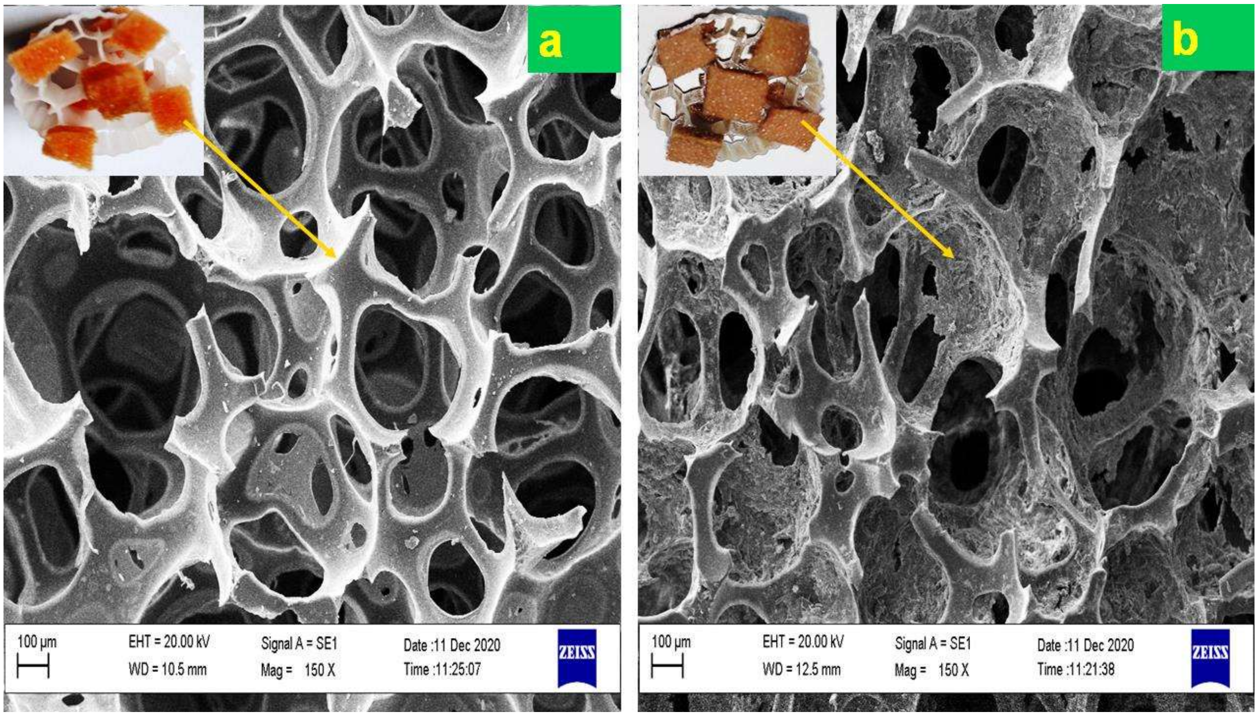

Figure. 4.3. SEM images of PP-PUF biocarrier used for CR dye biodegradation; (a) before immobilization (without bacterial adhesion), (b) after immobilization (with bacterial adhesion).

4.3.2. Effect of initial dye concentration on removal efficiency and elimination capacity

The MBBR was operated in a continuous mode at various ILRs (66 – 330.03 mg/L. d) by changing the initial CR dye concentration (50 – 250 mg/L) at a constant hydraulic retention time (HRT) of 18.18 h. The parameters, namely removal efficiency (RE) and elimination capacity (EC), were evaluated to analyze the effect of the initial dye concentration. The MBBR was operated at each specific condition until the steady-state outcomes were achieved, and the results were summarized in Table 4.2. After the start-up, the MBBR was fed synthetic wastewater at an initial CR dye concentration and ILR of 50 mg/L and 66 mg/L. d, respectively. The maximum RE and EC of 88.4 % and 58.34 mg/L. d, respectively, were found on the 17th day of operation. Then, the initial dye concentration was increased to 100 mg/L on the 18th day of operation. The MBBR attained the maximum RE on the 35th day of operation and was found to be lower than the previous condition. The maximum RE and EC were measured to be 80.8 % and 106.65 mg/L. d, respectively. A downward trend of RE and improvement of EC were found with the increase of initial dye concentration (Figure. 4.4). The ILR was further set at 198.01 mg/L. d by increasing the dye concentration to 150 mg/L. The RE was decreased due to the increase in loading rate, and the maximum RE was obtained to be 76.2 % on the 58th day of operation. However, the EC was enhanced due to increased substrate availability in the bioreactor and was found to be 150.88 mg/L. d. Similarly, the maximum RE of 66.4 to 64.64 % were evaluated at an initial dye concentration of 200 and 250 mg/L, respectively. The corresponding EC was measured to be 175.29 to 213.18 mg/L. d on the 113th day of operation.

In the present study, a low RE and high EC were found at a maximum initial dye concentration. Gwain et al. [37] have studied the effect of initial 4-chlorophenol (4-CP) concentration on the performance of an MBBR. The RE declined at a higher 4-CP concentration due to the washout of the substrate without complete degradation. A specific time period is always preferred for the metabolic activity of the microorganisms. The increase in dye concentration led to a higher ILR which caused an enhancement of substrate diffusion flux between the bulk liquid and biofilm (Mohanty and Kumar, 2021).

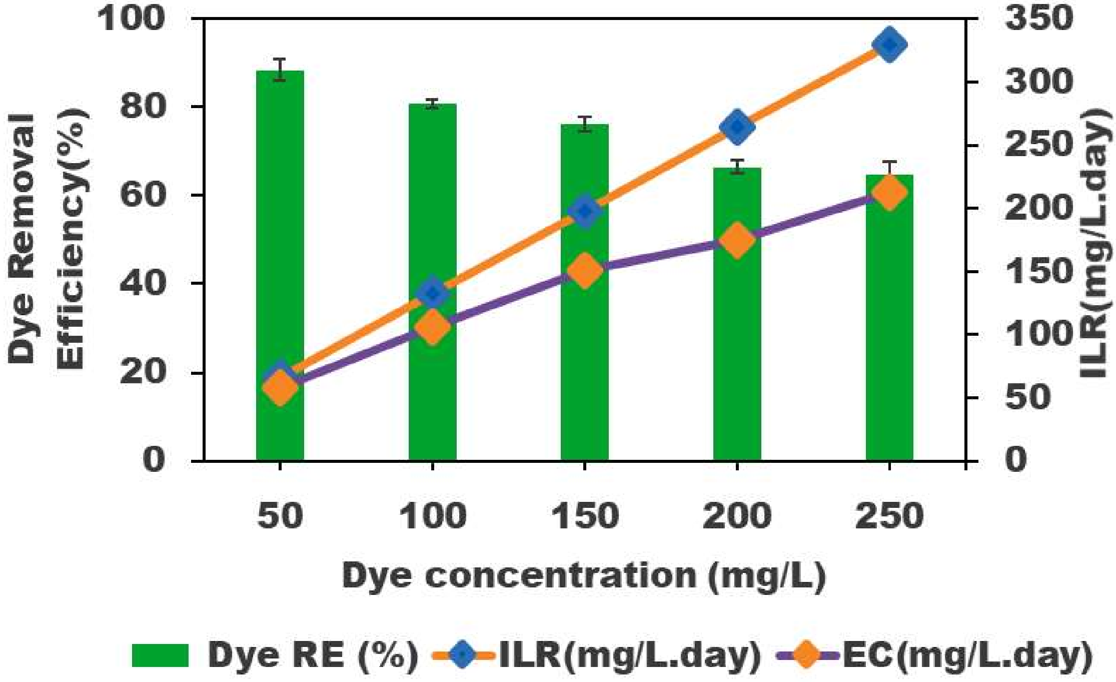

Figure. 4.4. Effect of initial dye concentration on dye removal efficiency and elimination capacity.

Table 4.2. Parameters obtained during the CR dye degradation in a continuous MBBR.

Process time (days)	Dye concentration (mg/L)	HRT (h)	ILR (mg/L. d)	Removal efficiency (%)	EC (mg/L. d)
1-17	50	18.18	66.0	88.4	58.34
18-35	100	18.18	132.01	80.8	106.65
36-58	150	18.18	198.01	76.2	150.88
59-82	200	18.18	264.02	66.4	175.30
83-113	250	18.18	330.03	64.6	213.18

HRT: Hydraulic retention time; ILR: Inlet loading rate; EC: Elimination capacity

4.3.3. Kinetic analysis during biodegradation of CR dye

4.3.3.1. First-order kinetic model

The straight-line graph was plotted between $\ln(\frac{D_t}{D_0})$ vs. time (t) and represented in Figure. 4.5. The First-order rate constants were evaluated from the slope of the curves and obtained to be 0.0397, 0.0368, 0.035, 0.0414, and 0.0381 hr^{-1} at initial CR dye concentrations of 50, 100, 150, 200, and 250 mg/L, respectively. The corresponding regression coefficients were found to be 0.97, 0.97, 0.96, 0.96, and 0.97, respectively (Table 4.3).

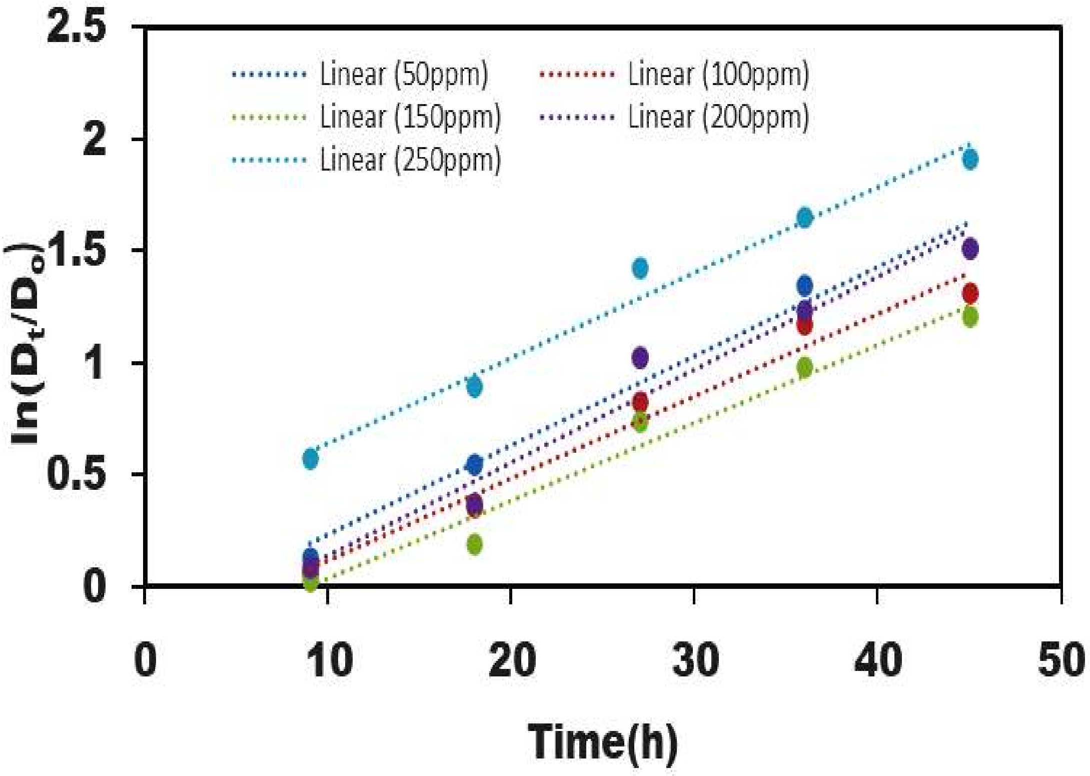

Figure. 4.5. First-order kinetic model for CR dye biodegradation.

4.3.3.2. Second-order kinetic model (Grau model)

The Second-order kinetic parameters were evaluated by plotting the graph between $(\frac{1}{D_t} - \frac{1}{D_0})$ and time (t) at each operating condition. The second-order kinetic constants were evaluated from Figure. 4.6 and obtained to be 0.002, 0.0008, 0.0005, 0.0005, and 0.0006 L/mg.h at initial CR dye concentrations of 50, 100, 150, 200, and 250 mg/L, respectively. The rate constants depend on dye concentration and available active bio-mass in the MBBR. The corresponding correlation coefficients (R^2) were obtained as 0.99, 0.98, 0.97, 0.97, and 0.98. The high values of R^2 (> 0.99) revealed better predictability of the experimental values.

The RMSE analysis of both the kinetic models was carried out to justify the significance of the statistical models, and the summary was provided in Table 4.3. A statistically significant model should have an RMSE value close to zero, showing a negligible difference between the predicted and experimental data (Huiliñir and Villegas, 2014; Sonwani et al., 2021). It was found that the values of RMSE are less for the second-order model compared to the first-order kinetic. Therefore, the second-order kinetic could be a suitable model for the prediction of CR dye utilization rate.

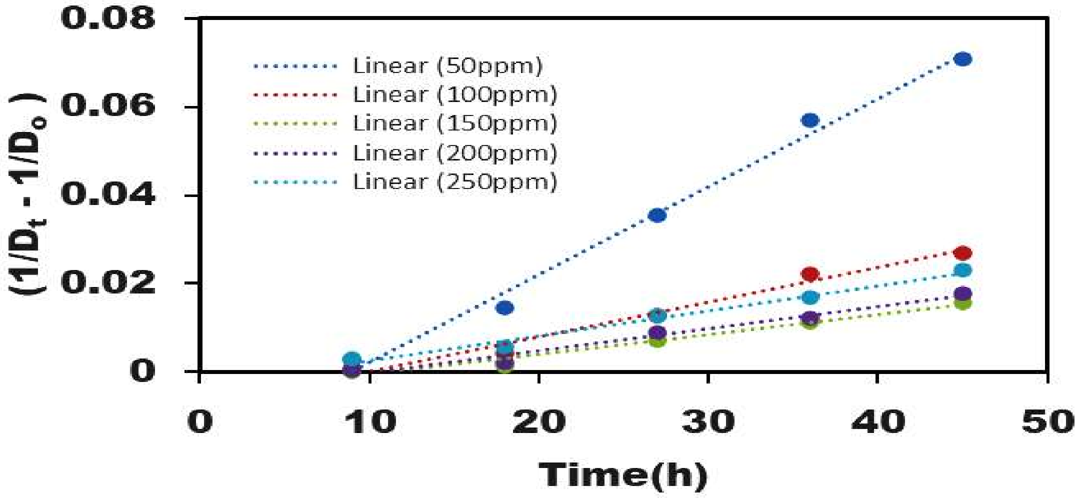

Figure. 4.6. Second-order kinetic model for CR dye biodegradation.

Table 4.3. Kinetic study of a first and second-order equation.

	First-order			Second-order		
Dye concentration (mg/L)	***K*** **(h^{-1})**	***R^2***	**RMSE**	***K_1*** **(L/mg. h)**	***R^2***	**RMSE**
50	0.0397	0.97	0.08445	0.002	0.99	0.00246
100	0.0368	0.97	0.07497	0.0008	0.98	0.00148
150	0.035	0.96	0.07977	0.0005	0.97	0.00170
200	0.0414	0.96	0.09792	0.0005	0.97	0.00096
250	0.0381	0.97	0.07335	0.0006	0.98	0.02610

4.4. Conclusion

In this study, the MBBR filled with isolated bacteria species (i.e., *Lysinibacillus fusiformis* KLM1 and *Lysinibacillus macrolides* KLM2) immobilized PP-PUF biocarrier showed a great extent towards the biodegradation of CR dye. The performance of MBBR was evaluated at various concentrations of CR dye (50 - 250 mg/L). The MBBR offered a maximum RE of 88.4 % at the initial dye concentration of 50 mg/L. Further, the dye RE was decreased with an increase in the initial concentration of CR dye. Two kinetics, i.e., First-order and Second-order models, were analyzed to estimate the substrate utilization kinetics in CR dye biodegradation. The high correlation coefficient ($R^2 > 0.97$) showed the high precision of the Second-order kinetic model during the biodegradation of CR dye in MBBR. However, the mass transfer phenomena and the enzymatic activity at a higher loading rate could be studied to enhance the present process. In conclusion, the current technology could be an economical and cost-effective method for biodegradation of textile effluents containing persistent azo dyes.