CHAPTER 4

A step towards enhancing the efficiency of biofilm mediated degradation of brilliant green dye in packed bed bioreactor: Statistical and toxicity analysis

4.1 Introduction

Textile and dyeing industries are considered the most polluting industries based on both the amount and the toxic content of effluents (Sonwani et al., 2021; Martinez-Lopez et al., 2019). Dye-contaminated effluents discharged from industries are considered one of the major concerns among environmentalists (Tian et al., 2021).

According to Martinez-Lopez et al. (2019) about 100000 tons of dyes contaminated effluents are discharged into the environment every year globally. Most of the azo dyes are recalcitrant, carcinogenic, xenobiotic, and mutagenic and comprises of high suspended solids and unpleasant odor (Rajasimman et al., 2017; Maurya et al., 2021; Singh et al., 2014). Therefore, the remediation and abatement of these toxic dyes from the environment is a serious concern since these poses adverse impacts on humans as well as on the aquatic ecosystem (Bharti et al., 2019).

The physical and chemical techniques, such as adsorption, electrochemical oxidation, photocatalytic degradation, and advanced oxidation processes have been widely used to handle azo dyes in the last three decades (Monira, 2022). These processes have limited application due to their high cost and hazardous by-product formation (Abu Talha et al., 2018; Wang et al., 2009). Among all these methods, biological treatment (i.e., biodegradation) is the most suitable, energy-

efficient, and environmentally friendly option that can be used in industries at various scales (Wang et al., 2021; Ledakowicz et al., 2017; Sonwani et al., 2021). Sludge produced by biological processes is relatively less harmful to the environment compared to physical and chemical processes (Bharti et al., 2019). Various researchers adopted the free cell technique for dye degradation, which involves the addition of free cells microorganisms directly to the bioreactor (Sonwani et al., 2020b). However, free cells processes often have limited application under a high inlet loading rate (Sonwani et al., 2019). To rectify the above problem, the immobilized cell technique has received major attention. In this technique, the microorganisms grow on a carrier surface, and subsequently immobilized carriers are used as packing material in the bioreactor (Padmanaban et al., 2016; Sonwani et al., 2020b). In this direction, Packed bed bioreactors (PBBRs) are widely employed due to their low cost, high specific area, and long retention time (Abu Talha et al., 2018).

In connection with the optimization and process designing of bioreactor, RSM is one of the useful statistical experimental designs and provides information about the combined effect of various parameters (Ba and Boyaci, 2007; Zhao et al., 2017). However, the major issue in the treatment of dye-containing wastewater by biological process is the nonlinear nature of the process, modeling of such complex process cannot be successfully done by RSM. To rectify these limitations, ANN can be used to approximate the functions (Ghaedi and Vafaei, 2017; Khataee et al., 2010; Zafar et al., 2012).

ANN is a computational-based model which is influenced by biological neural processing, which can be used to model highly nonlinear processes. ANN has intrinsic characteristic like learning and works in two manners which can be classified as training mode and normal mode. For the development of an ANN model, the experimental dataset of RSM is itself adequate. ANN could

be advantageous over RSM as it does not require preparatory specification of fitting function (Desai et al., 2008; Ghaedi et al., 2014; Pakravan et al., 2015).

The objective of this study was to instigate the individual and interactive effects of key process parameters (pH, dye concentration, and inoculum dose) on biodegradation of BG dye in PBBR and comparative toxicity assessment. PUF was used as packing material which was immobilized with *Bacillus licheniformis* ST5. The performance of PBBR was evaluated under various inlet loading rates. The final results predicted by both RSM and ANN were compared using error functions such as root mean square error (RMSE), mean absolute error (MAE), standard error of prediction (SEP), bias (B_f) and accuracy (A_f) factors, and relative importance of each input parameters was calculated using Garson's method. Monod and Andrews-Haldane models were used for the evaluation of kinetic parameters. *Vigna radiata* seeds were used for physiological assessment, including photosynthetic pigment content and antioxidant enzyme activity. *P. Luminescence* bacteria was used for acute and chronic toxicity assessment.

4.2 Materials and method

4.2.1 Experimental setup and immobilization procedure

The experimental setup mainly consists of bioreactor (i.e., PBBR), peristaltic pump (Miclins PP 10), feed tank, rotameter (Flow India), and air pump (XP-AC-24L) (**Fig. 4.1**). PBBR is made of borosilicate glass (height = 100 cm; diameter = 5 cm) having total capacity of 1.9 L. The working capacity of PBBR was 1 L. Polyurethane foam (PUF) was used as packing material to immobilize the microorganisms. PUF sheet was acquired from a local shop in Varanasi and cut into cubes (1 cm × 1 cm × 1 cm). Before the immobilization process, PUF cubes were washed with 70% ethanol and further by sterilized distilled water and dried at 50°C in an oven. For the

immobilization purpose, acclimatized microbial culture (1.1× 10^7 CFU/mL) was inoculated on PUF carrier and reactor was run on batch mode for 15 days with MSM containing glucose (3 g) and microbial culture to ensure the formation of biofilm on PUF surface (Swain et al., 2021a). Biofilm developed on PUF carrier was visually analyzed by optical microscopy (Nikon ECLIPSE Ni-E, Japan).

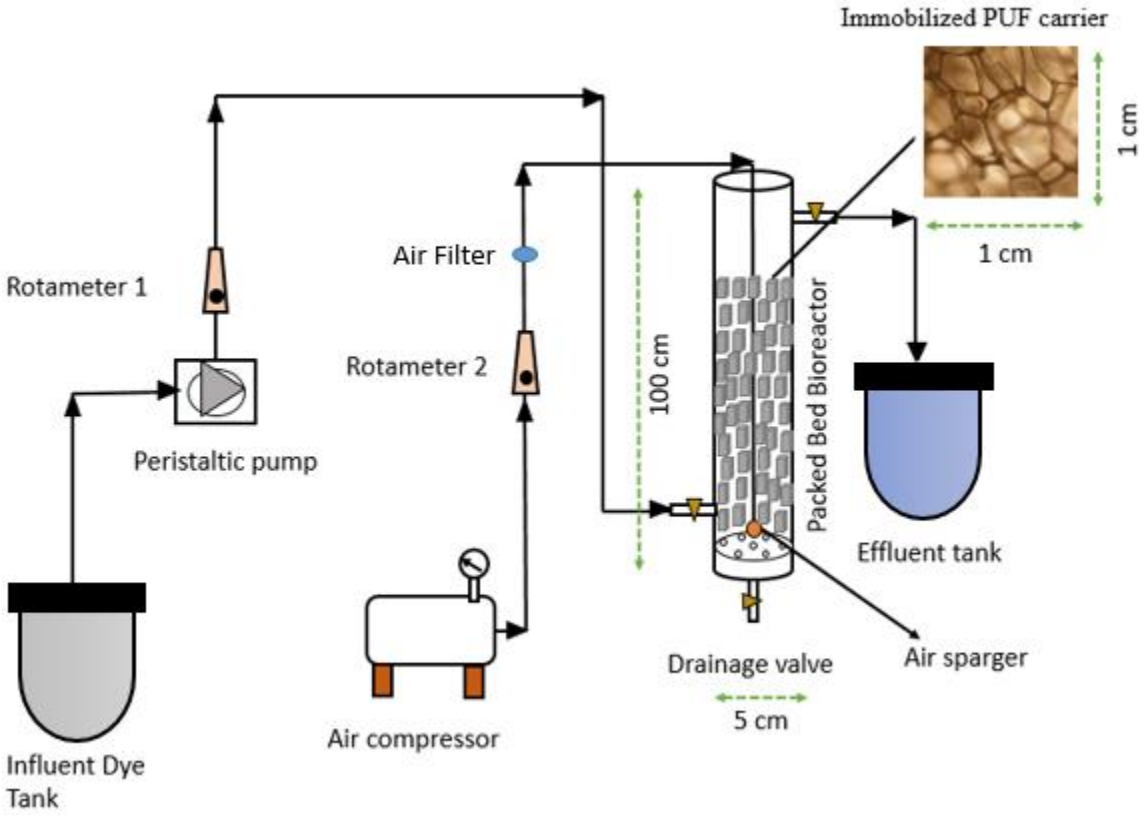


Fig. 4.1 Schematic diagram of PBBR for degradation BG dye.

4.2.2 Biofilm Growth evaluation

Generally, biofilm formation takes place when microorganisms shift from a planktonic state to a sessile state (Coffey and Anderson, 2014). Structurally, biofilm systems are defined as microorganisms and extracellular polymeric substances (EPS) associated with them. These EPS are mainly composed of protein, polysaccharides, and DNA, which provide resistance to external

stress. In this study, we have evaluated biofilm growth efficiency for different concentrations of BG dye. Firstly, the bacterial culture was grown planktonically to a stationary phase in nutrient broth (NB) at 35℃ overnight. Then overnight culture was diluted 1:100 into fresh NB for biofilm assay. 100 μL of the freshly inoculated sample was then transferred to 96 well microtiter plate with varying BG dye concentration and incubated at 35℃±1.0 for 48 h to facilitate the biofilm formation. After incubation, the microtiter plate was washed 3 times with MilliQ water, followed by 30 min drying. The well was then dyed for 30 minutes with 1% crystal violet (CV) and rinsed with distilled water once more and dried overnight (Otoole and Kolter, 1998). For the quantification of that stained biofilm, 150 μL of 30% acetic acid was added to solubilize the CV. After 15 min of incubation, each sample had its absorbance measured at 600 nm in a microtiter reader (Synergy H1).

4.2.3 Optimization by RSM (BBD)

RSM is a statistical technique used for modeling and analysis of the functional relationship between design variable and response variable (Gusain et al., 2016; Roosta et al., 2014; Sharma et al., 2009). This method has been widely utilized to examine how several process variables such as pH, dye concentration, inoculum dose, and temperature (Ravi Kumar Sonwani et al., 2019). Box-Behnken design (BBD) is considered an effective form of RSM and is used by various researchers to evaluate the effect of process parameters on dye degradation (Das and Mishra, 2017; Haq et al., 2018; Sharma et al., 2009).

In this study, a three-level three-factor BBD was used to scrutinize and validate process parameters influencing the biodegradation of BG dye. Analysis was executed using Design expert 12 software

(Stat Ease, trail version). Process variables and their coded range are shown in **Table 4.1**. The total number of experiments (*N*) based on this design is defined as

$$N = 2k(k-1) + C_0 \quad (4.1)$$

where *k* represents the number of independent variables and C_0 represents number of central points.

The relevant model term was examined using a second-order polynomial model equation expressed in Eq. (4.2).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (4.2)$$

where *Y* represents response variable (% degradation of dye), β_0 is the constant term, β_1 , β_2 and β_3 coefficient of linear effect terms β_{11}, β_{22} and β_{33} coefficient of square effect term, $\beta_1 \beta_2$, $\beta_1 \beta_3$ and $\beta_2 \beta_3$ coefficient of interaction effect term. All experiments were performed according to BBD and a total 15 set of experiments were performed in triplicates. The response (% Degradation) of dye was investigated by pred. R^2, adj. R^2, ANOVA and response plot (3D surface plot and contour plot).

Table 4.1 Independent variables and their coded value used in BBD

Variables	Factors	Range and level(Coded)		
		-1	0	+1
pH	A	5	7	9
Dye concentration (mg/L)	B	50	100	150
Inoculum dose (mL)	C	2.5	5	7.5

4.2.4 Modeling by ANN

The human brain, which has billions of neurons coupled together to comprehend a wide variety of complicated information, is an inspiration for ANN. A computational neural network comprises of an input layer of neurons (i.e., independent variable), several hidden layers, and an output layer (i.e., dependent variable) as shown in **Fig 4.2.** Each layers are connected together by different weight connections. The input layer gets data from outside sources and send it to the hidden layer for processing. In order to connect the input and output layers, the hidden layer's function is necessary. The artificial neurons in hidden layer acts like biological neurons in the brain as it processes its probabilistic input and converts them into an output. A three-layered feedforward with back propagation neural network (3:10:1) was utilized for modeling of biodegradation process.

In this study, the input subjected to the neural network model were similar to the parameter used in RSM model, i.e., pH of mixture, initial dye concentration, inoculum dose, and the output (i.e., response) was considered as %dye degradation. A tan-sigmoidal transfer function was utilized as an activation function between input and hidden layer. For network training, the Levenberg-Marquardt back propagation technique was used. The data set (comprised of 15 datapoints) was divided into three parts, 70% of data used for training (11 data points), 15% of the data used for validation (2 data points) and 15% used for the test (2 data points). All the calculations for ANN were performed using Neural Network Toolbox of MATLAB Software Version 7.9 (R2009b).

The RSM and ANN results were compared statistically and the parameter for the comparison include error functions such as RMSE, SEP, MAE, and MPE along with bias (B_f) and accuracy (A_f) factors calculated using Eq. (4.3) – (4.8) (Zafar et al., 2012) .

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(Y_{i,e} - Y_{i,p}\right)^2} \quad (4.3)$$

$$SEP(\%) = \frac{RMSE}{Y_e} \times 100 \quad (4.4)$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}\left(Y_{i,e} - Y_{i,p}\right) \quad (4.5)$$

$$MPE(\%) = \frac{100}{n}\sum_{i=1}^{n}\left[\frac{\left(Y_{i,e} - Y_{i,p}\right)}{Y_{i,p}}\right] \quad (4.6)$$

$$B_f = 10^{\left(\frac{\sum_{i=1}^{n}\log\frac{Y_{i,p}}{Y_{i,e}}}{n}\right)} \quad (4.7)$$

$$A_f = 10^{\left(\frac{\sum_{i=1}^{n}\left|\log\frac{Y_{i,p}}{Y_{i,e}}\right|}{n}\right)} \quad (4.8)$$

where *n* represents the number of data, $Y_{i,e}$ indicates the experimental value, $Y_{i,p}$ indicates the predicted value, and Y_e indicates the mean value of the experimentally determined.

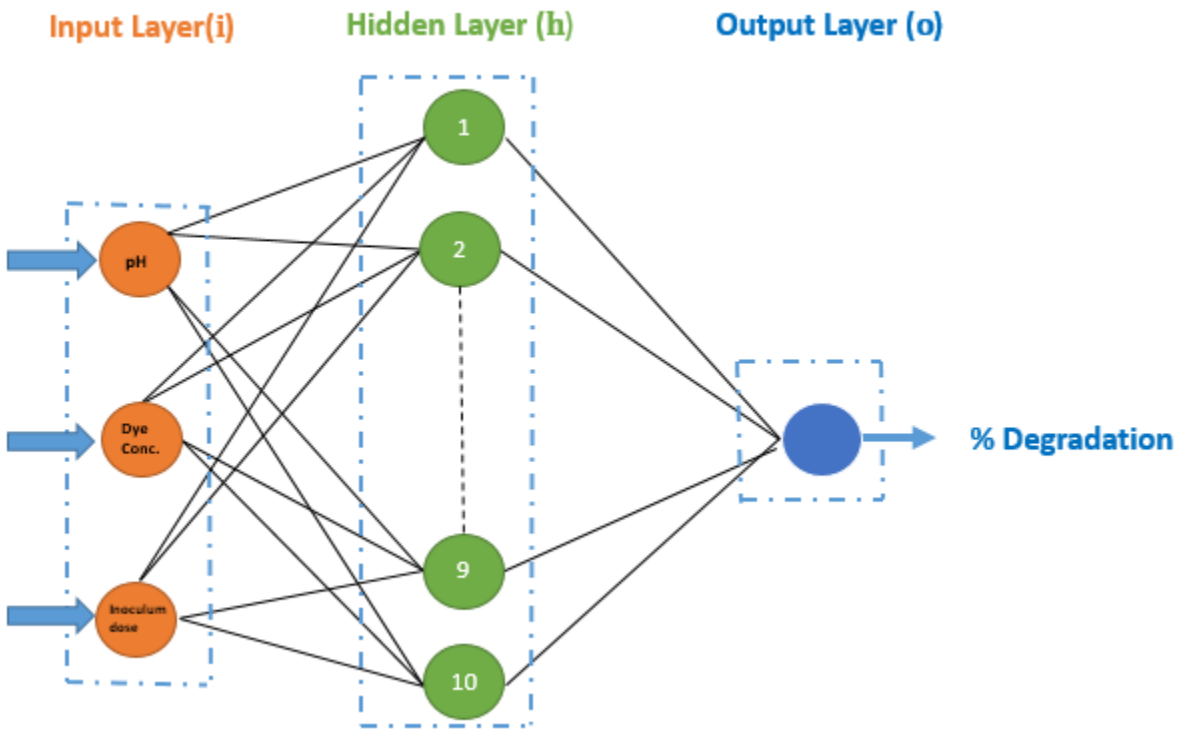


Fig. 4.2 The optimized ANN structure for removal of BG dye

4.2.5 Garson's method

Garson's method was used to estimate the level of influence of parameters on BG dye biodegradation. Relative importance was calculated using Eq.(4.9), which utilizes the connection weight of ANN (Estahbanati et al., 2017; Khan et al., 2020).

$$I_j = \frac{\sum_{m=1}^{m=N_h}\left(\left(\left|w_{jm}^{ih}\right|/\sum_{k=1}^{k=N_i}\left|w_{km}^{ih}\right|\right)\times\left|w_{mn}^{ho}\right|\right)}{\sum_{k=1}^{k=N_i}\left\{\sum_{m=1}^{m=N_h}\left(\left(\left|W_{km}^{ih}\right|/\sum_{k=1}^{k=N_i}\left|W_{km}^{ih}\right|\right)\times\left|W_{mn}^{ho}\right|\right)\right\}} \quad (4.9)$$

Where I_j is the importance of the j[th] input variable on output variables, W represents the link weights, and N_i and N_h are the numbers of input and hidden neurons. The superscripts 'i', 'h' and 'o' refer to input, hidden, and output layers respectively.

4.2.6 Continuous study of removal of BG in PBBR

The optimized value obtained from ANN is used to assess the performance of PBBR under continuous mode for different inlet flow rates (10-50 mL/h). The arrangements were made in the bioreactor to add nutrient solution continuously. Aerobic conditions were maintained in PBBR by continuously supplying air through a sparger (Geed et al., 2017; Kureel et al., 2017). The performance of PBBR was evaluated using the following parameters:

$$\text{Removal efficiency (RE \%)} = \frac{C_i - C_o}{C_i} \times 100 \quad (4.10)$$

$$\text{Elimination Capacity (EC, mg/L.d)} = \frac{(C_i - C_o) \times Q}{V_R} \quad (4.11)$$

$$\text{Inlet loading rate (ILR, mg/L.d)} = \frac{C_i \times Q}{V_R} \quad (4.12)$$

where C_i and C_o are inlet and outlet concentration of BG dye (mg/L), Q is the volumetric flow rate (mL/h), and V_R is the working volume of the reactor (L).

4.2.7 Kinetics study

The various kinetic models were notably used to assess the effectiveness of the bioreactor by figuring out the substrate utilization and biodegradation rates. The kinetic rate was predicted by Non-inhibitory Monod and inhibitory Haldane-Andrews models (Pandey and Sarkar, 2019).

Monod model has been selected for fitting the experimental data (degradation of BG dye). The equation can be expressed as:

$$\mu = \frac{1}{X}\frac{dx}{dt} = \frac{\mu_{max} S}{K_s + S} \quad (4.13)$$

where μ is specific growth (per day), K_s *i*s half-saturation constant (mg/L), *S*, *X*, and *t* are initial substrate, microbial cell concentration (mg/L), and time (day), respectively.

This equation can be rewritten as:

$$\mu = \frac{log\frac{X_t}{X_o}}{t-t_o} \quad (4.14)$$

where X_t and X_o is microbial cell concentration at time t and t_o, respectively.

Due to substrate inhibition, the Monod model is unable to predict the specific growth rate of microorganisms at higher loading rates, hence Haldane-Andrews kinetics was selected as a replacement.

The equation includes an inhibitory constant (K_i)

$$\mu = \frac{\mu_{max}S}{K_s+S+\frac{S^2}{K_i}} \quad (4.15)$$

where K_i is inhibition constant (mg/L).

4.3 Results and discussion

4.3.1 Identification of dye degrading bacterial strain

The bacterial strain which has the potential to degrade BG dye was isolated from dye contaminated site. Based on DNA sequencing, the strain was identified as *Bacillus licheniformis*. After that, the sequence was submitted in GenBank database of NCBI and obtained an accession number (MZ220456). Phylogenetic tree of *Bacillus licheniformis* ST5 (MZ220456) has been shown in **Fig. 4.3**. Several *bacillus* strains, namely *Bacillus subtills*, *Bacillus sp.* (MH587030.1),

and *Brevibacillus parabrevis* have been employed to degrade a wide range of dyes (Abu Talha et al., 2018; Sharma et al., 2009; Sonwani et al., 2020b).

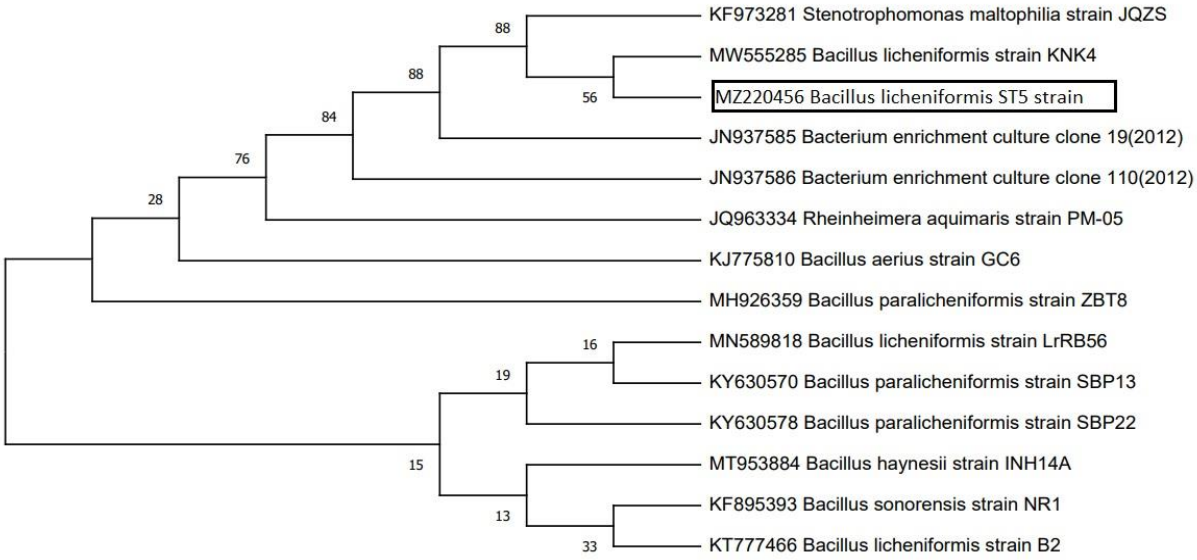


Fig. 4.3 Phylogenetic tree of bacterial strain *Bacillus licheniformis* ST5 (MZ220456) isolated from dye contaminated site**.**

4.3.2 Morphological analysis of PUF carrier and biodegradation study

The morphological study of the PUF carrier was carried out using a microscope (**Fig 4.4**). The microscopic image of the control carrier (0^{th} day) represents that several small micropores were found onto the surface of the carrier. These micropores provide a large surface area for the development of biofilm. However, a dense layer of biofilm was developed on the PUF carrier after fifteen days, which confirms the successful immobilization of biofilm on the carrier.

In batch studies, the effect of biosorption and biodegradation on BG dye removal using living (i.e., immobilized) and non-living (i.e., autoclaved) bacterial species was examined. The non-living culture shows very less removal of dye (50 mg/L) and only 5.2% removal obtained, which is

probably due to biosorption. The living culture showed 88% removal of BG dye under similar conditions. From the above analysis, it can be concluded that the removal of BG dye occurs primarily due to biodegradation (Swain et al., 2021a).

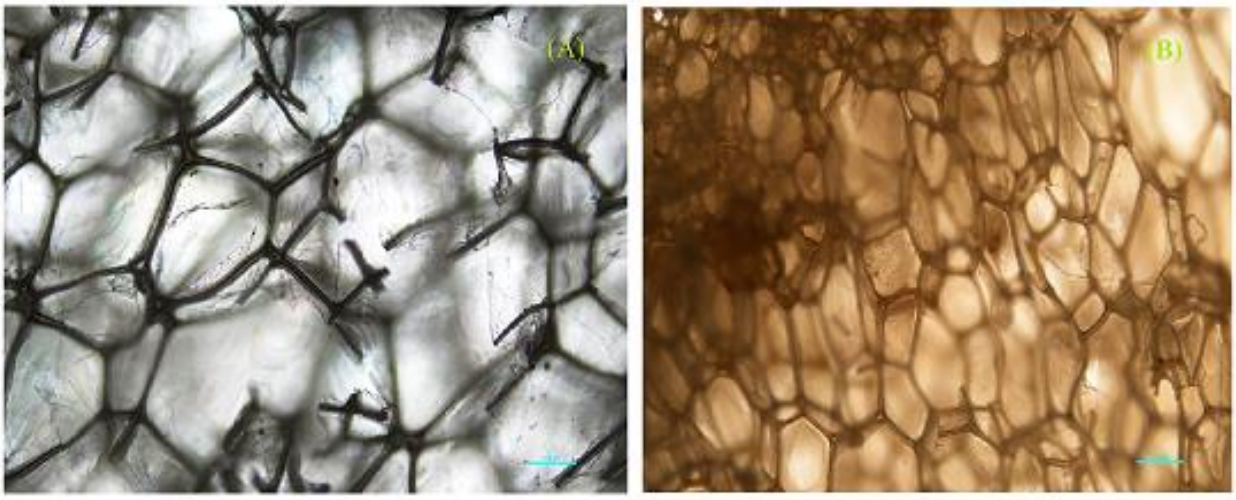


Fig. 4.4 Microscopic image of packing material (A) at 0^{th} day (B) after 15^{th} day.

4.3.3 Effect of process parameters on BG dye degradation

In order to assess the effect of dye concentration on the BG dye degradation, a comparative study was performed with various dye concentration (50–200 mg/L) under optimum conditions (pH = 8.0, Temperature = 35℃, Inoculum dose = 7.5 mL). **Fig 4.5**(a) shows that up to 150 mg/L, bacteria successfully degraded the BG dye but beyond this, the degradation started to decrease. This indicates the possibility of inhibition, which can also be supported by biofilm growth study (Padmanaban et al., 2016). According to **Fig 4.5**(b), pH also has a greater influence on dye degradation. As we increased the pH of the solution from 5.0–9.0, dye degradation also increased. Dye adsorption was inhibited at low pH because of greater competition between H^+ ions and the cationic BG ions (Torbati, 2016). The temperature was assessed at 25℃, 35℃, and 45℃ for an initial dye concentration of 100 mg/L. **Fig. 4.5**(c) shows that dye degradation was maximum at 35

°C. It could be attributed to the fact that most favorable temperatures for biochemical processes are between 30–35°C. The effect of inoculum dose on dye degradation was also assessed for three different doses 2.5 mL, 5.0 mL, and 7.5 mL. From **Fig. 4.5**(d), it is clearly visible, as we increased the inoculum dose, the dye degradation was also increased. The explanation of this observation could be that with an increase in inoculum dose, the number of active cells also increased, which leads to an increase in dye degradation (Khataee et al., 2010).

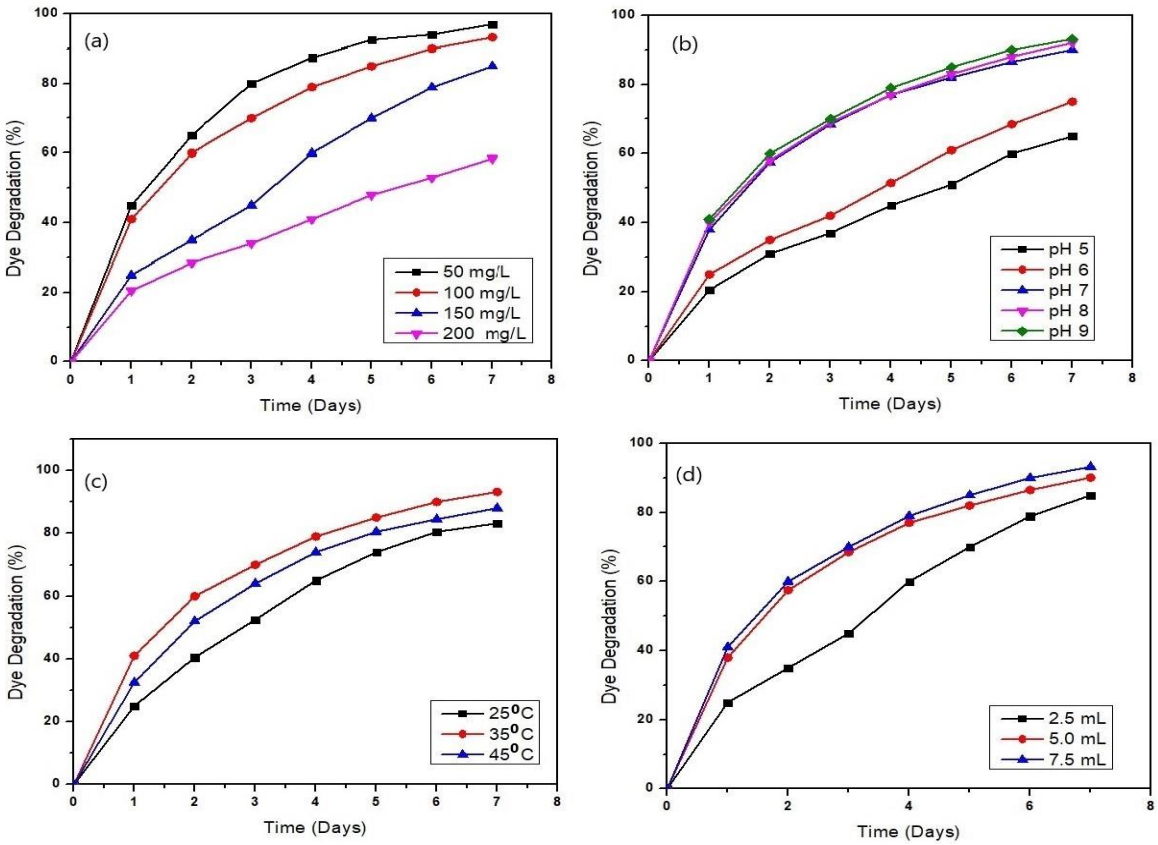


Fig. 4.5 (a) Effect of different dye concentration on the biological removal of BG dye (pH = 8.0, Temperature = 35°C, Inoculum dose = 7.5 mL). (b) Effect of different pH on the biological removal of BG ($[BG]_0$ = 100 mg/L, Temperature = 35°C, Inoculum dose = 7.5 mL). (c) Effect of temperature on the biological removal of BG dye (pH = 8.0, $[BG]_0$ = 100 mg/L, Inoculum dose = 7.5 mL). (d) Effect of different inoculum dose on the biological removal of BG dye (pH = 8.0, $[BG]_0$ = 100 mg/L, Temperature = 35°C).

4.3.4 Impact of BG dye on biofilm growth

CV staining method was utilized for the quantification of biofilm growth. The results show that a lower concentration of BG dye enhanced the biofilm growth, whereas high concentrations (200–250 mg/L) inhibited the biofilm growth (**Fig. 4.6**). Contrarily, it has been suggested that the dyes ability to stimulate microbial breakdown through the formation of reactive oxygen species and DNA destruction may explain why biofilms inhibited at greater dye concentrations (Barathi et al., 2022b). The fact that dispersed biofilm could grow even at a high concentration of BG dye (250 ppm) suggested that the small amount of bacteria can withstand the toxicity of high concentrations of dye to produce biofilm.

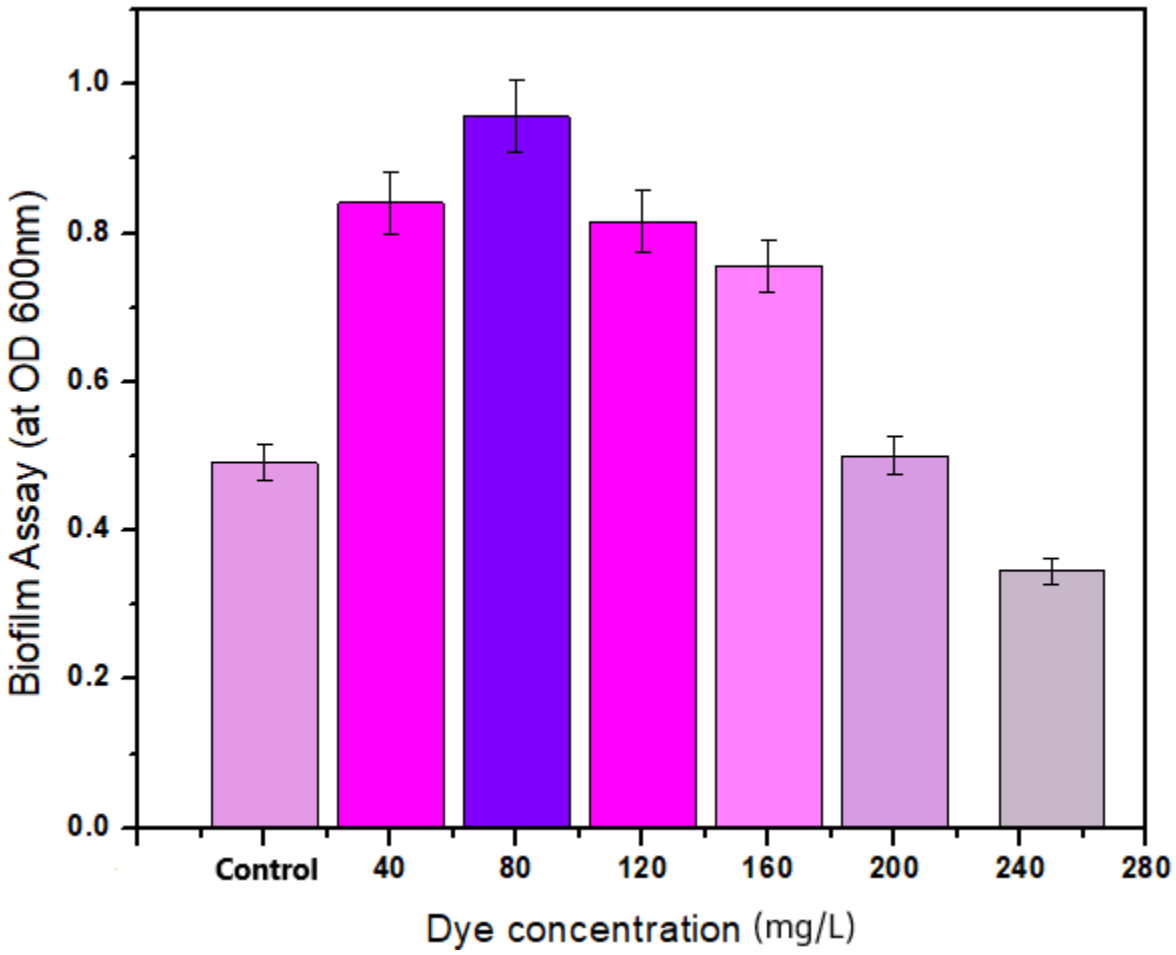


Fig.4.6 Effect of BG dye concentration on biofilm growth.

4.3.5 RSM Model Analysis

The biodegradation studies on the decolonization of brilliant green dye were performed based on BBD of RSM and obtained results are presented in **Table 4.2**. An empirical relationship between the response (% degradation) and independent variable has been obtained in the form of second-order polynomial, and is given by Eq. (4.16).

$$\%\ \text{BG degradation} = 85.0 + 11.69A - 3.75B + 3.86C + 0.725AB + 1.2AC - 0.075BC - 9.75A^2 + 2.28B^2 - 1.95C^2 \qquad (4.16)$$

where *A* represents the pH of the mixture, *B* represents initial dye concentration (mg/L), and *C* represents the inoculum dose (mL). From ANOVA analysis, it was observed that a low probability value ($p<0.0001$) indicates the model is significance with data. The suitability of the model can be confirmed by the value of regression coefficient R^2, adj. R^2, Pred. R^2. ANOVA analysis for experimental results of BBD is represented in **Table 4.3** data. In the present work, the values of R^2, adj. R^2, and Pred. R^2 were found to be 0.995, 0.986, and 0.923, respectively. The value of R^2 (0.9952) revealed the significance of the model, and only 0.48% of the total variation cannot clarify by the model. The value of adj. R^2 (0.9866) is in reasonable convention with Pred. R^2 (0.9232). The value of lack of fit ($p>0.05$) indicates the significance of the quadratic model.

Table 4.2 BBD matrix of independent variables (coded) and their corresponding responses.

Run	A (pH)	B (Dye Conc.)	C (Inoculum dose)	Response	Predicted Value byRSM	Predicted Value ByANN
1	1	-1	0	92.1	91.99	92.22
2	0	-1	-1	84	84.89	83.94
3	-1	-1	0	71.4	70.44	71.16
4	-1	1	0	61.5	61.74	61.42
5	0	0	0	85	84.9	84.98
6	1	1	0	85.1	86.06	85.09
7	0	0	0	85	84.9	84.98
8	0	1	1	86.5	85.61	84.69
9	-1	0	-1	59	59.08	59.03
10	0	-1	1	93.2	93.51	93.19

11	1	0	1	90	89.34	90.01
12	1	0	-1	81.2	80.54	81.02
13	-1	0	1	63	65.66	61.98
14	0	0	0	85	84.9	84.98
15	0	1	-1	77.6	77.29	77.58

Table 4.3 ANOVA results of BBD model for degradation of brilliant green dye.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1724.99	9	191.67	115.22	< 0.0001	significant
A-pH	1092.78	1	1092.78	656.92	< 0.0001	
B-Dye Concentration	112.50	1	112.50	67.63	0.0004	
C-Inoculum dose	119.35	1	119.35	71.75	0.0004	
AB	2.10	1	2.10	1.26	0.3120	
AC	5.76	1	5.76	3.46	0.1218	
BC	0.0225	1	0.0225	0.0135	0.9119	
A^2	351.00	1	351.00	211.00	< 0.0001	
B^2	19.11	1	19.11	11.49	0.0195	
C^2	14.04	1	14.04	8.44	0.0336	

4.3.6 Interactive effect of process variables on dye degradation

A 3-D plots were constructed to investigate the combined effect of pH, initial dye concentration,

and inoculum dose.

4.3.6.1 Effect of pH of mixture and initial dye concentration

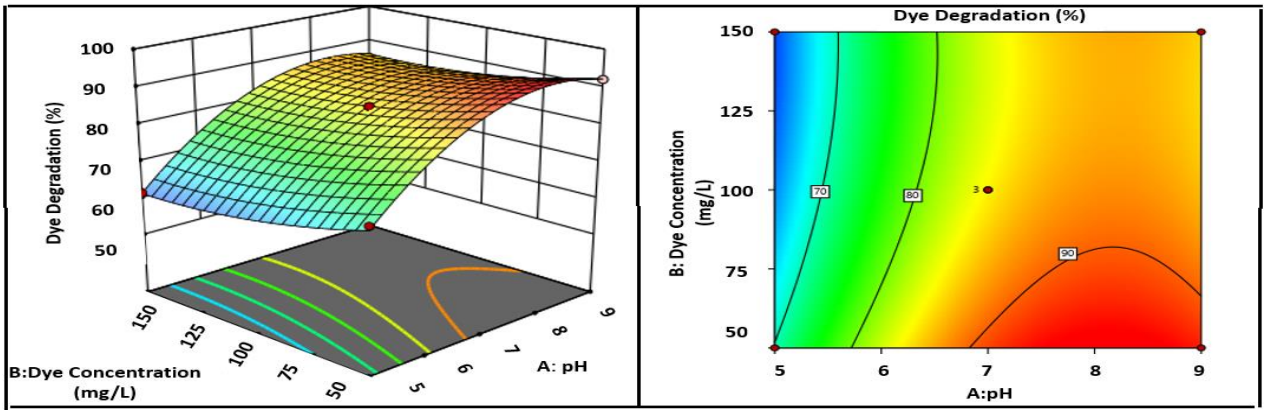
The collective effect of both parameters is represented by 3-D surface and contour plots (**Fig 4.7**(a)). It was found that the pH of the medium had a significant impact on the degradation of the dye. As we varied the pH of the solution from acidic to alkaline, the degradation increased. At pH 9.0, 92.1% of dye degradation was observed at 50 mg/L of dye concentration, which was reduced to 71.4% at pH 5.0 under the same conditions. A similar trend can also be seen for higher dye concentration (150 mg/L), where 85.1% degradation was achieved at pH 9.0, which was reduced to 61.5% at pH 5.0. The enzymatic activity of microorganisms is affected in alkaline and acidic conditions, which significantly reducing the overall process efficiency (Sonwani et al., 2020b).

4.3.6.2 Effect of pH of mixture and inoculum dose

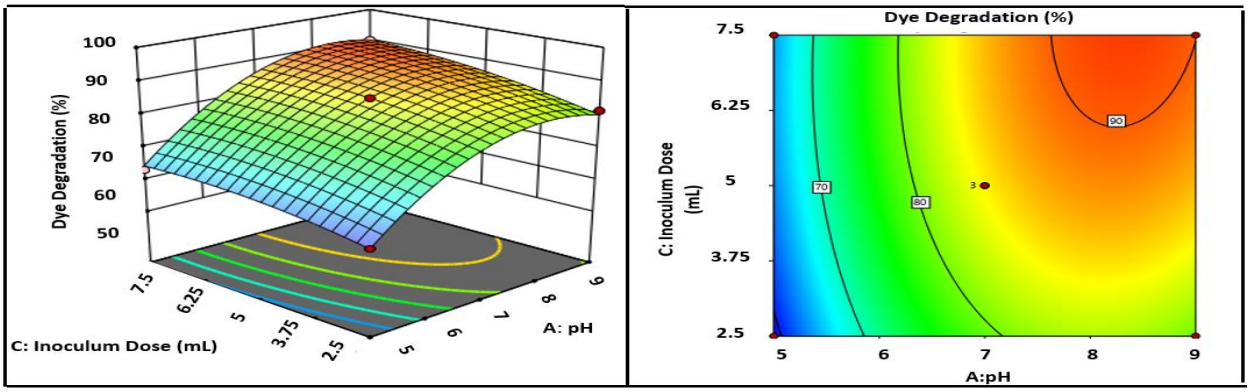
The effect of the pH of solution and inoculum dose on degradation was represented by 3-D surface plot (**Fig 4.7**(b)). It was found that the degradation of BG dye removal is greatly influenced by the pH of the solution and the bacterial inoculum. The plot reveals that 81.2% dye degradation was obtained at high pH (9.0) and low inoculum dose, (2.5 mL) and degradation was increased to 90% at an inoculum dose of 7.5 mL for a constant dye concentration of 100 mg/L. This may be concluded as inoculum dose up to optimum value have a significant effect on degradation. As the solution pH was decreased to 5.0, dye degradation was also decreased to 59% for the same inoculum dose and dye concentration (7.5 mL and 100 mg/L). Since the bacteria used in this study is an alkaliphile, lowering the pH to acidic can lead to the denaturation of proteins and destroy their activity.

4.3.6.3 Effect of initial dye concentration and inoculum dose

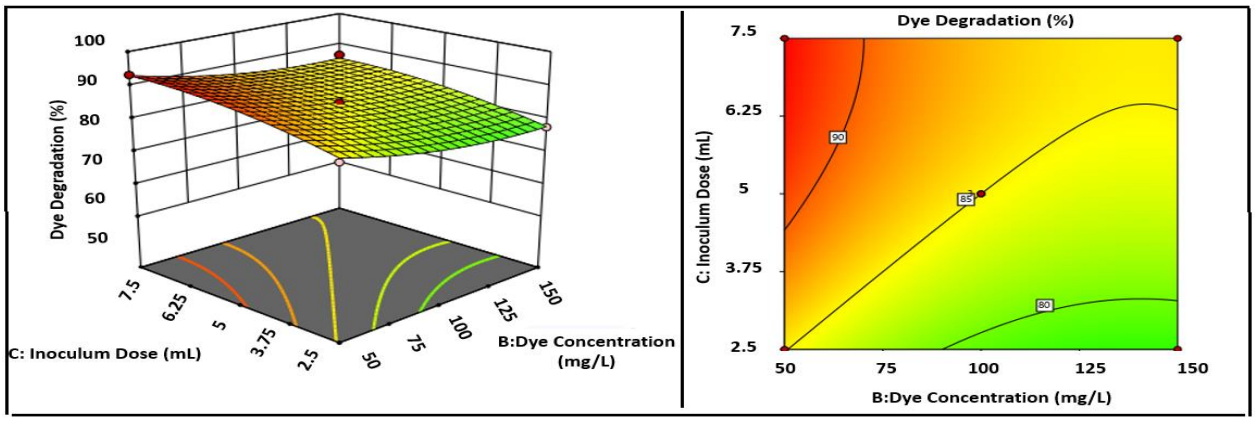
The collective effect of initial dye concentration and inoculum dose on decolorization was represented by 3-D surface plot (**Fig 4.7**(c)). The plot clearly indicates that as the dye concentration was raised from 50 mg/L to 150 mg/L, the degradation decreased from 84% to 77.6% at an inoculum dose of 2.5 mL and pH of 7.0. Similarly, at pH 9.0, the degradation decreased from 92.1% to 85.1% as we increased the dye concentration from 50 mg/L to 150 mg/L. The decrease in degradation at high concentrations of BG dye was due to dye toxicity and substrate inhibition (Sharma et al., 2009). As we increased the inoculum dose from 2.5 mL to 7.5 mL, the degradation significantly increased from 84% to 93.2% at pH of 7.0, and dye concentration of 50 mg/L. Similarly, at high pH (9) value also as we increased the value of inoculum dose from 2.5 mL to 7.5 mL, the degradation was also increased from 81.2% to 90% at dye concentration of 100 mg/L. Similar kind of trend was also reported by Krishnan et al., 2017 for the degradation of direct blue-6 and direct black-19 dye.



(a)



(b)



(c)

Fig. 4.7 3-D response surface and contour plot for %degradation of brilliant green dye (a) effect of pH and dye concentration (b) effect of pH and inoculum dose (c) effect of dye concentration and inoculum dose

4.3.7 Modeling by ANN

ANN is widely accepted powerful tool for the simulation and optimization of complex and ill-defined problems (Torbati, 2016; Venkatesh Prabhu et al., 2016). The topology of the present ANN model involves input (3 neurons), hidden (10 neurons), and an output layer (1 neuron). This ANN topology entitled as multilayer perceptron, MLP (3:10:1). The RSM data was used for this purpose. Utilizing the Levenberg-Marquardt back propagation algorithm, the network was trained. As an activation function for hidden layers, a tan-sigmoid transfer function was utilized. The data was further split into three subsets which included 70% of the data for training (11 data), 15% of

the data for validation (2 data), and 15% of the data for the test (2 data). The plot for output (% degradation) with respect to validation, training, and test data are shown in **Fig 4.8**. From the experimental and predicted data, it was observed that the response predicted by the ANN model was highly significant and a high correlation coefficient (0.999) indicates the effectiveness of the ANN model.

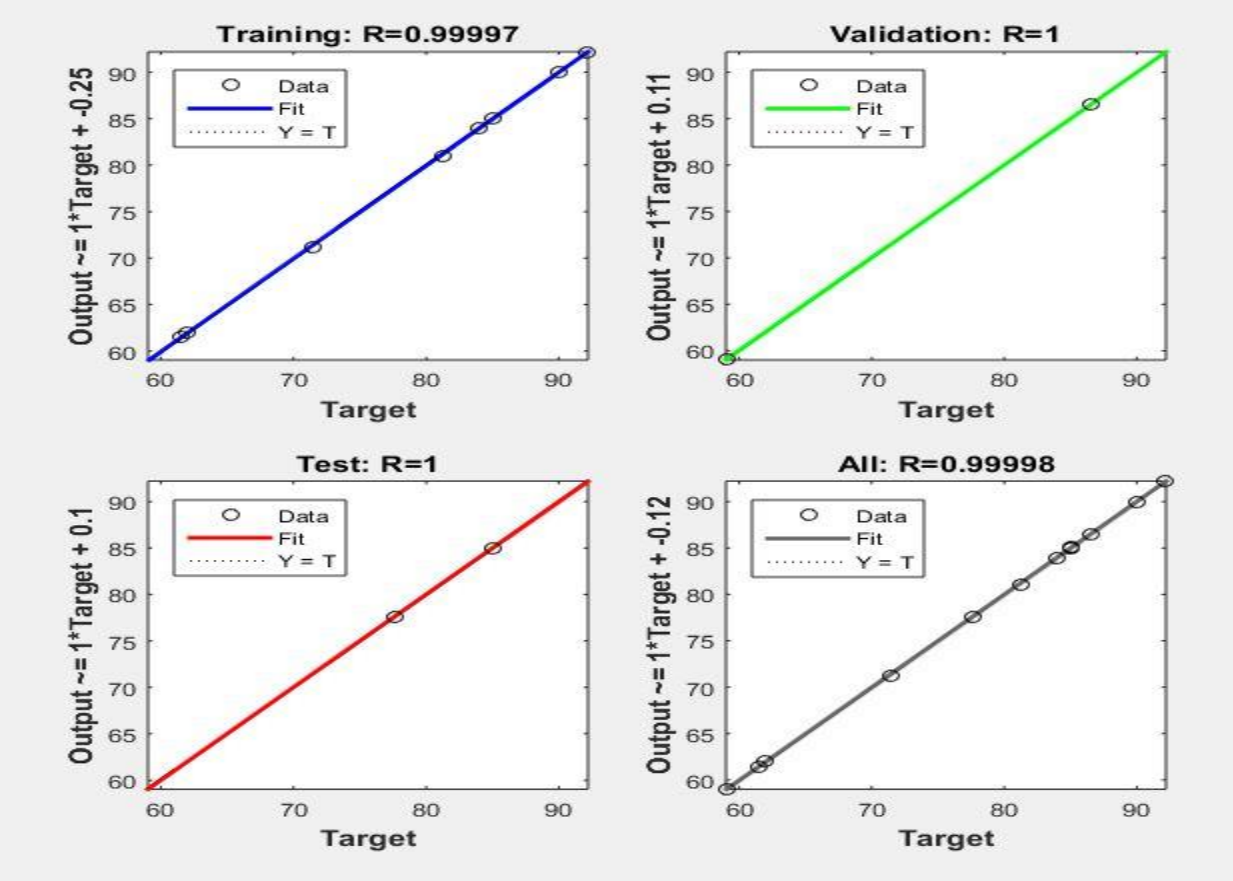


Fig. 4.8 Neural Network plot with respect to training, validation and test dataset for % degradation of BG dye.

4.3.8 Comparison of optimization Models

The RSM and ANN results were compared statistically and the parameter for the comparison include error functions such as RMSE, SEP, MAE, and MPE. **Table. 4.4** shows that

the value generated from the ANN model are relatively closer to the experimental value than the RSM model. Furthermore, the value of B_f and A_f for both models approaches unity, indicating good agreement between anticipated and experimental values. The plot shown in **Fig. 4.9** clearly demonstrates that % degradation predicted using ANN was almost similar to the experimental degradation. **Fig. 4.10** compares the residuals distribution between the experimental and RSM and ANN values. It is concluded that the accuracy of the prediction of ANN is more than that of the RSM model for highly nonlinear processes.

Table 4.4 Comparison between ANN and RSM

	%Decolorization	
Statistical parameter	RSM	ANN
RSME	0.8638	0.270
SEP (%)	1.081	0.338
MAE	0.133	0.0979
MPE (%)	0.2066	0.148

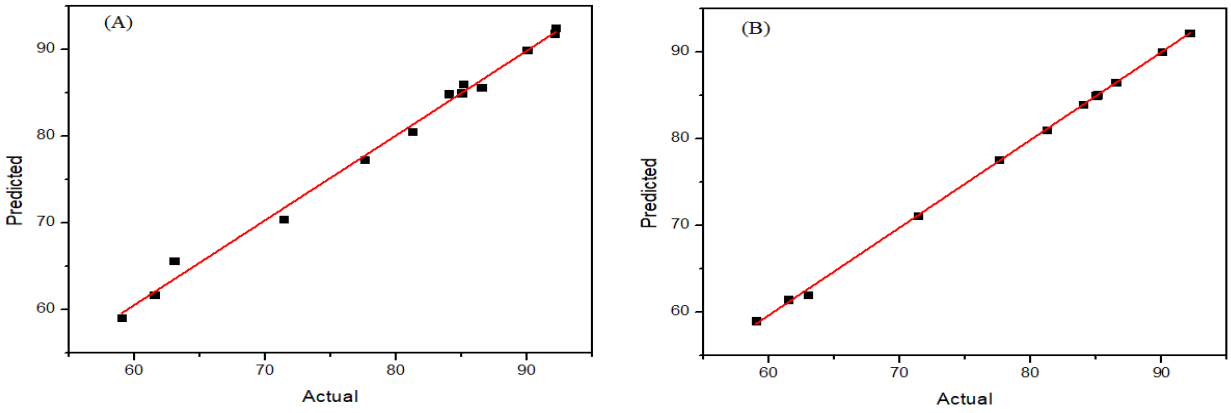


Fig.4.9 Graph of theoretical %degradation vs predicted %degradation (A) Using RSM (B) Using ANN

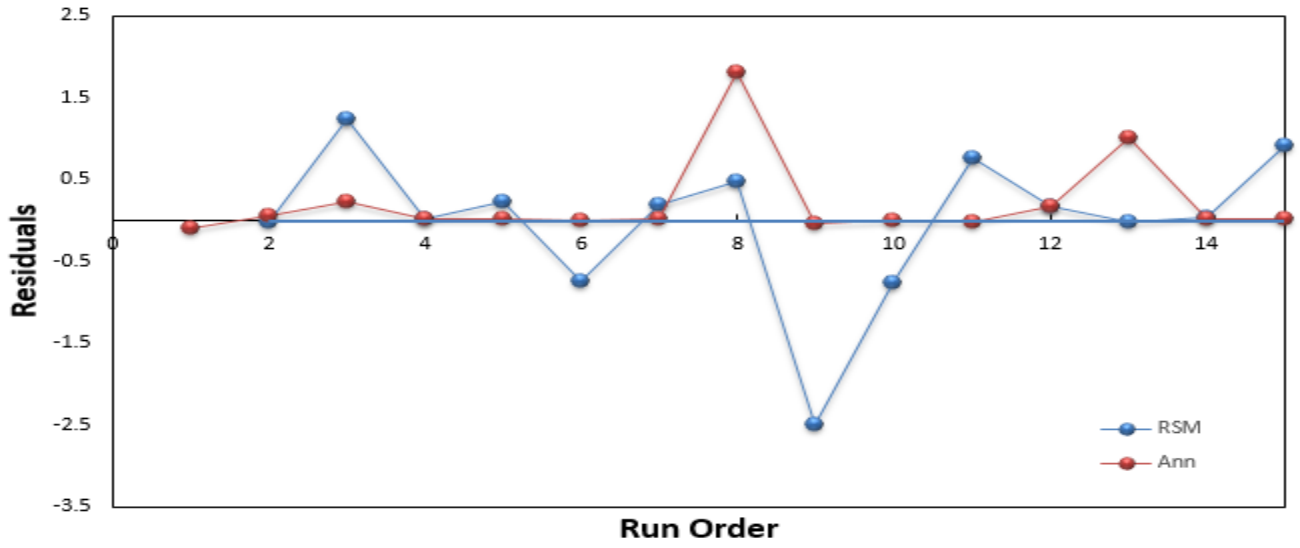


Fig. 4.10 Residual Distribution of RSM and ANN models.

4.3.9 Comparative importance of operating parameters

Garson's method was utilized to estimate the level of influence of operating parameters on the biodegradation of BG dye. Connecting the weight value of the model enlisted in **Table 4.5** data. The importance of input parameters evaluated by Eq. 4.9 is illustrated in **Fig. 4.11**. It can be noted that all the parameters (pH, dye concentration, inoculum dose) have a major effect on biodegradation efficiency. However, pH has a greater effect among these parameters with a relative importance of 46.0%. The enzymatic activity of microorganisms is significantly impacted by alkaline and acidic environments, as previously mentioned, which makes pH a stronger parameter of relative importance.

Table 4.5 Linked weight between input to hidden layer (W^{ih}) and hidden to output layer (W^{ho})

	Input layer to Hidden layer (W^{ih})			**Output Layer (W^{ho})**
Neuron	pH	dye concentration	Inoculum dose	% degradation
1	1.6324	-2.4127	0.85329	0.73163
2	-2.3943	-1.8195	-0.22171	0.011004
3	1.6177	-1.0895	2.3017	0.23595
4	-1.7168	1.7079	1.75	-0.047977
5	-0.7782	-2.8517	-1.14814	-0.21586
6	-1.6851	-0.86806	2.2622	0.64092
7	-0.64908	-1.7927	2.3096	-0.69969
8	0.55895	2.9036	0.004351	-0.30503
9	-2.9068	-0.23224	-1.0807	-1.1744
10	-1.8312	2.6104	0.54458	-0.44613

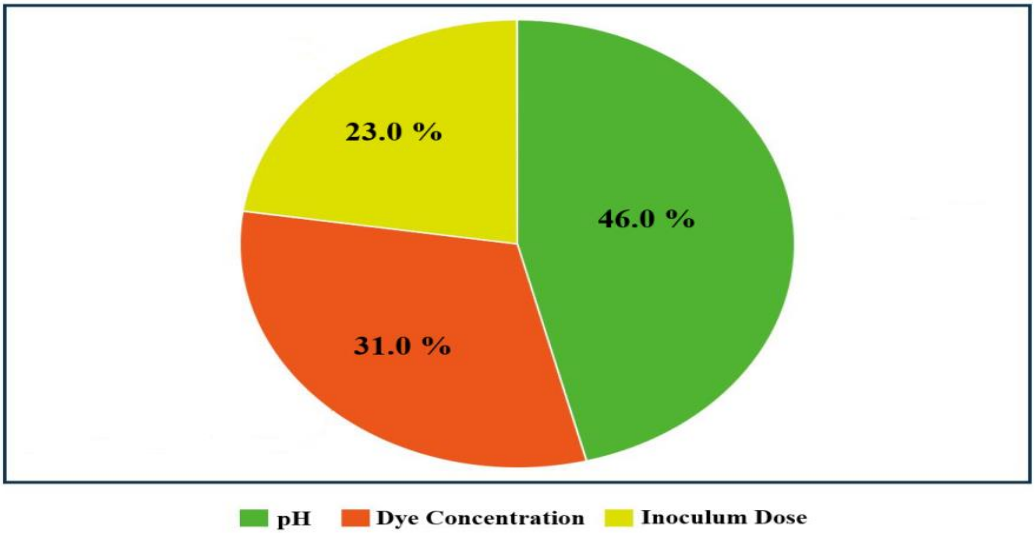


Fig. 4.11 The relative importance (%) of input parameters of ANN model for the biodegradation of BG dye.

4.3.10 Continuous study in PBBR for BG dye biodegradation

BG dye biodegradation was investigated in continuous PBBR at various flow rates (10–50 mL/h) under optimum condition and different parameters were evaluated to assess the performance of the bioreactor (**Fig. 4.12**). Initially, the feed flow rate was maintained at 10 mL/h, inlet concentration was 100 mg/L, and corresponding ILR was 24 (mg/L.d). The maximum value of EC (21.88 mg/L.d) was achieved in 15 days of operation and RE was 91.2%. On 16^{th} day, flowrate was increased to 20 mL/h, corresponding EC was 34.2 mg/L.d. A sharp reduction in RE was observed on 16^{th} day, but it improved gradually and attained a steady-state value of 86.7% on 27^{th} day, and the corresponding EC was 41.61 (mg/L.d). On 28^{th} day the flow rate was further increased to 30 (mL/h), corresponding ILR to 72 (mg/L.d). A similar trend was observed in RE, which again stabilizes on 49^{th} day (74.4%). The maximum EC of 88.08 mg/L.d was observed at 50 mL/h flow rate, corresponding RE was 73.4%. The final dye RE was reduced from 91.2 to 73.4%, with an increasing flow rates from 10 – 50 (mL/h). From **Fig. 4.12**, it is clearly visible that at high flowrate

(50 mL/h), RE was reduced. This phenomenon was observed in bioreactors due to change in rate control mechanism (Geed et al., 2017; Maurya et al., 2021; Sonwani et al., 2020b).

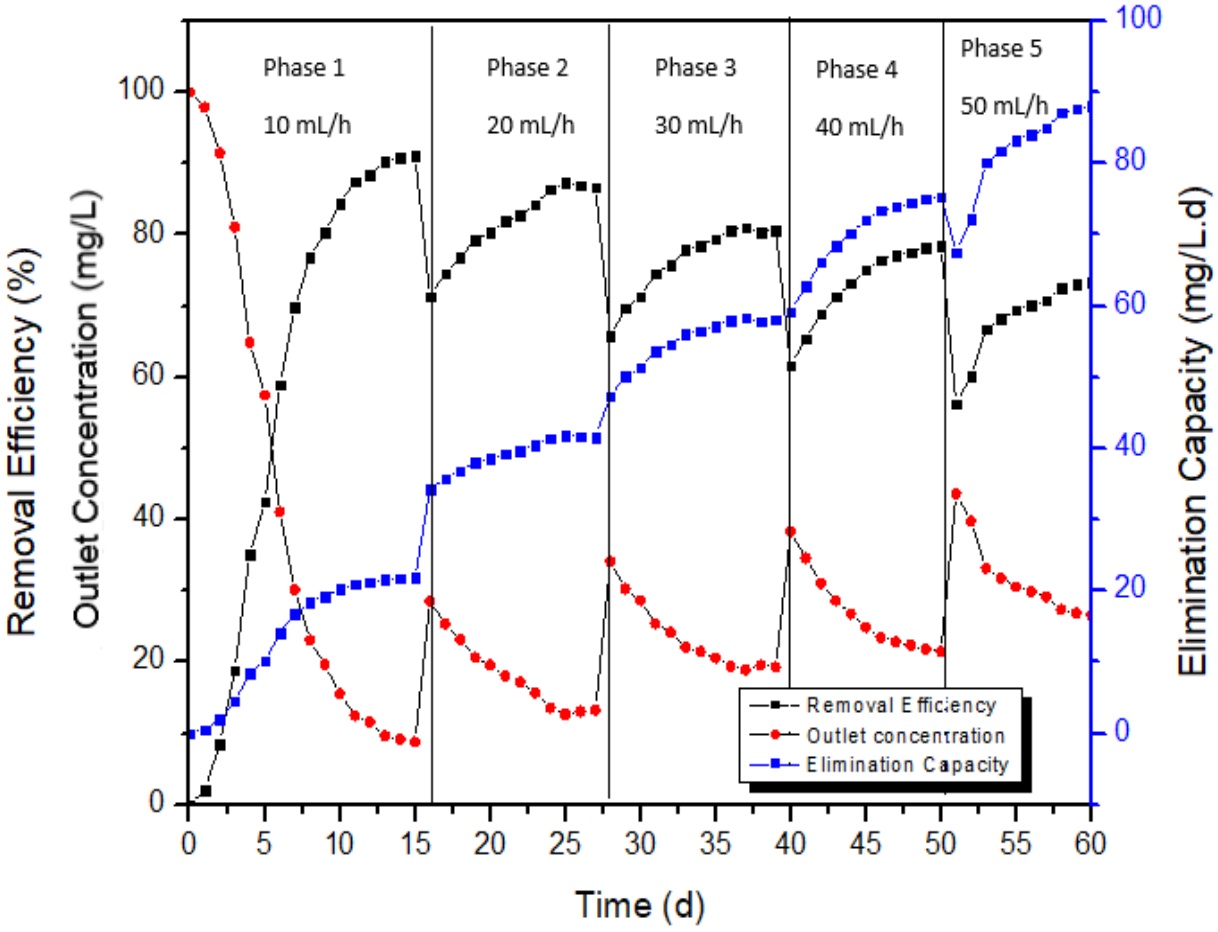
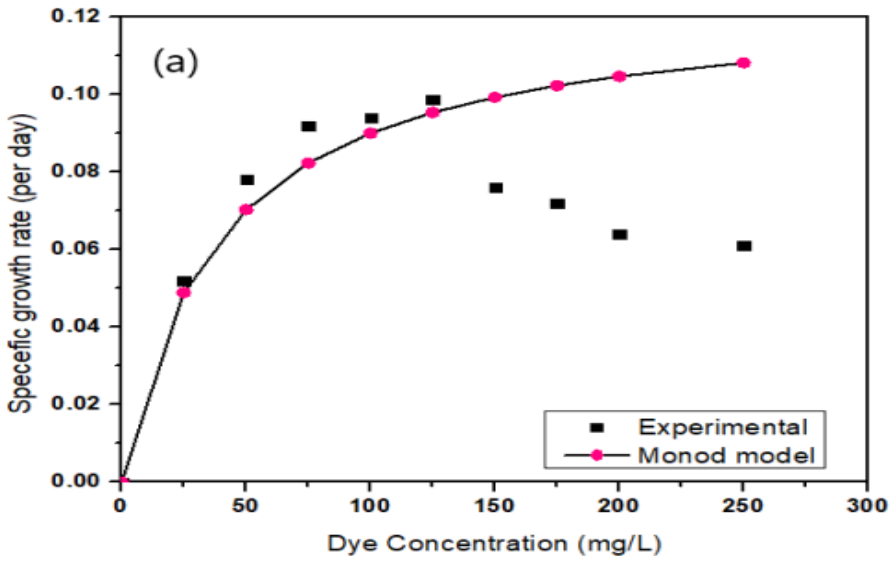


Fig. 4.12 Effect of various inlet flowrate on removal efficiency and elimination capacity for performance of PBBR.

4.3.11 Kinetic study

The experimental data of substrate concentration (i.e., BG dye) and specific growth rate plotted for the Monod and Andrews-Haldane model are shown in **Fig. 4.13**. With the increase in the initial concentration of dye, the specific growth rate was initially increased up to 125 mg/L of dye concentration. Beyond this, the value decreased the specific growth rate. The kinetic

parameters value; μ_{max} and K_s of Monod model were obtained as 0.125 (per day) and 38.9 mg/L, respectively. The high value of μ_{max} and low value of K_s is favorable for any biodegradation. The value of μ_{max}/K_s was found to be 0.00388 L/mg.day. The kinetic parameters for Andrews-Haldane model i.e., K_s, μ_{max}, and K_i were obtained as 45.1 mg/L, 0.175 per day, and 170 mg/L, respectively with regression coefficient of 0.986. The value of K_i indicates the inhibition tendency of substrate (Jegan et al., 2010). The low value of K_i indicated that the biomass has lower resistance to substrate inhibition. The summary of kinetic parameters obtained by both models is shown in **Table. 4.6.**



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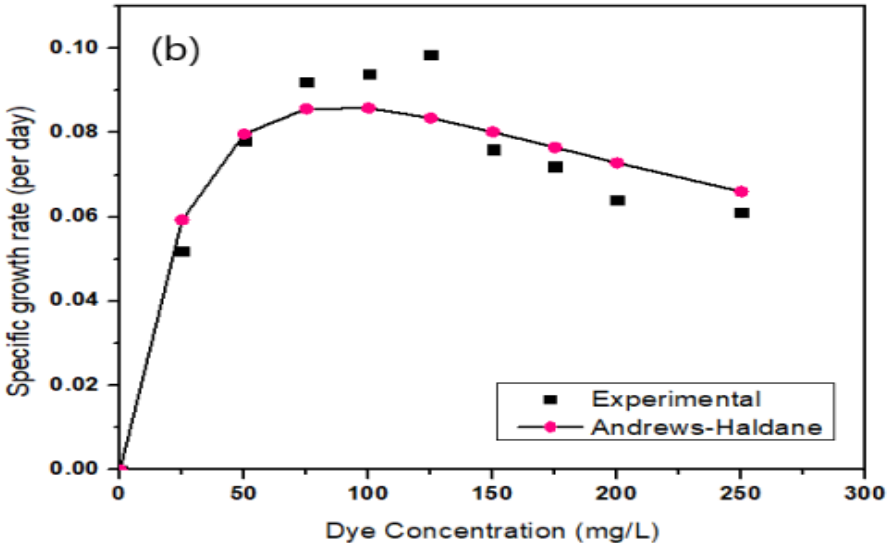

Fig.4.13 Experimental and predicted plot fitted by (a) Monod model (b) Andrews- Haldane model.

Table 4.6 Summery of Kinetic parameters for degradation of BG dye

Dye Concentration (mg/L)	Model	μ_{max} (per day)	K_s (mg/L)	K_i (mg/L)	R^2
0-250	Monod	0.125	38.9	-	0.94
0-250	Andrews-Haldane	0.175	45.1	170	0.98

μ_{max}: Max specific growth (per day); K_s : Half-saturation constant (mg/L); K_i : inhibition constant (mg/L).

4.3.12 FTIR analysis of control and degraded samples

The KBr disc method was used to perform FTIR analysis on BG dye before and after treatment. A significant change in functional groups of control and biologically treated samples confirms the biodegradation of BG dye (**Fig. 4.14**). FTIR spectra of the control sample showed

peak at 3430 cm^{-1} attributed to stretching of O−H group, a peak at 1645 cm^{-1} due to C=N stretching. A peak at 1380 cm^{-1} shows strong S=O stretching of sulfonic acid. The spectra analysis of the biologically treated sample shows major shifts between 1500 cm^{-1} to 1000 cm^{-1} and there is a disappearance of peak at 1380 cm^{-1}. A peak at 920 cm^{-1} showed the aromatic nature of amines. The difference between the FTIR spectra of the control and biologically treated samples indicates that the BG dye in PBBR was successfully degraded.

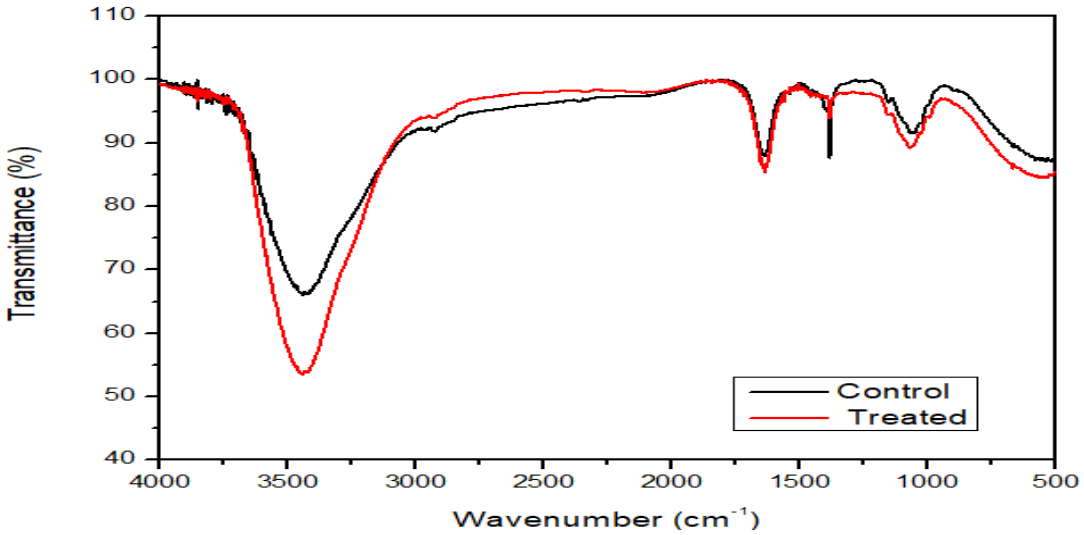

Fig. 4.14 FTIR spectrum of BG dye before and after treatment in PBBR

4.3.13 Toxicity analysis

4.3.13.1 Plant pigment content

The plant pigment content was evaluated for *Vigna radiata* seed after exposing it to DW, UT, and BT samples for 10 days. According to **Fig. 4.15(a)**, it is clearly visible that pigment content (chlorophyll a, b, and carotenoid) was decreased after being exposed to UT and BT solution. Chlorophyll content of the UT sample was reduced up to 2 times compared to the control DW sample. Thus, it may be assumed that the presence of a high concentration of BG either

prevented the biosynthesis of the aforementioned pigments or facilitated their degradation (Torbati, 2016).

4.3.13.2 SOD enzymatic activity

One of the important enzymes that get engaged in cellular defense against reactive oxygen species in living beings is superoxide dismutase (SOD). The phototoxic effects of the UT and BT wastewater on the activities of the SOD enzyme for root and shoots germination of *Vigna radiata* are depicted in **Fig. 4.15(b)**. It is clearly visible that the SOD activity of UT sample was increased up to 5 times compared to the BT and DW sample. The enhanced production of superoxide anions in the UT wastewater caused an increase in the quantity of SOD as a defense mechanism of the *Vigna radiata* seeds, resulting in a drastic increase in SOD activity. For the BT sample, SOD activity was significantly reduced but remained substantially different from the control.

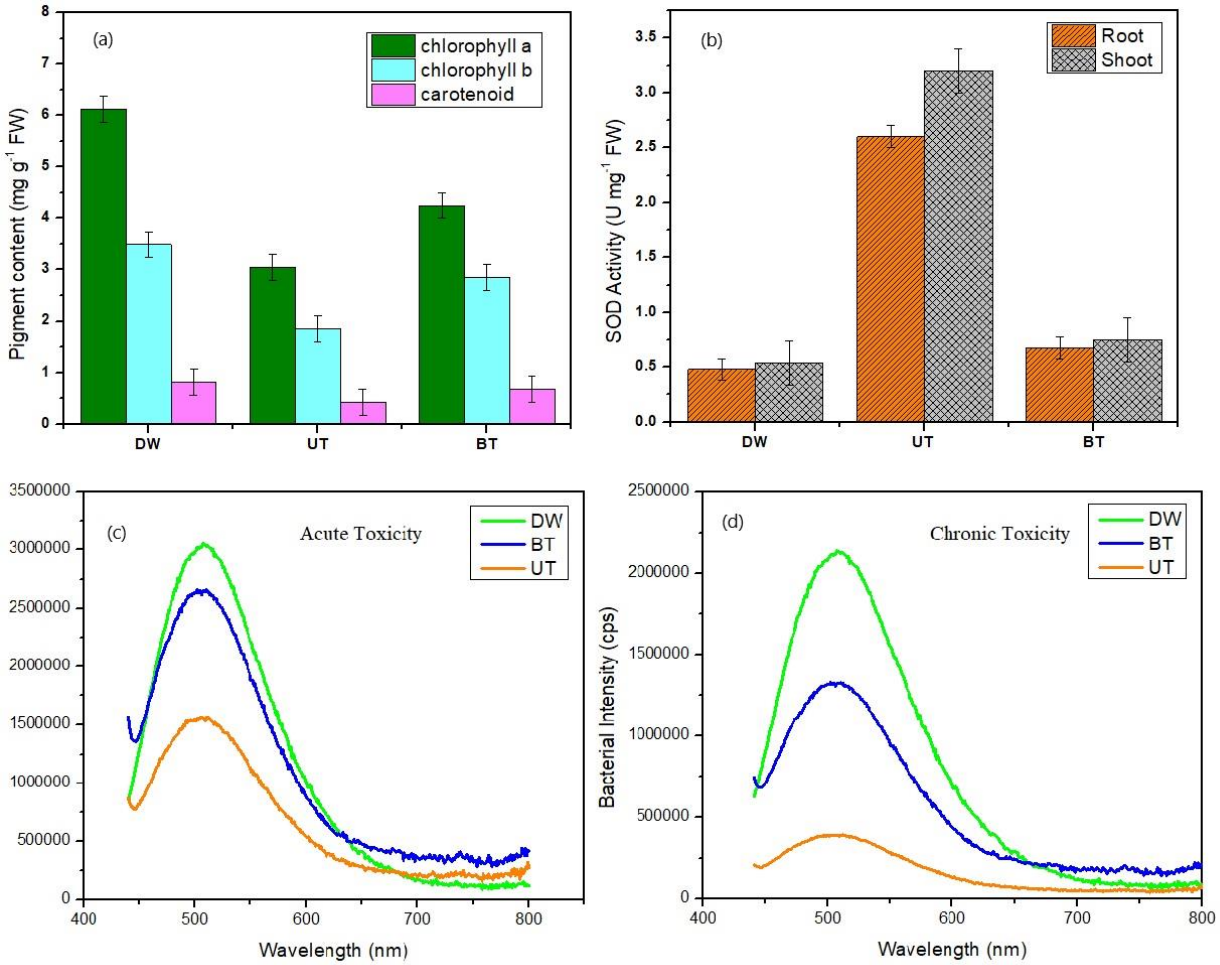

Fig. 4.15 (a) Content of Chlorophyll a, b, and carotenoid (mg g-1 FW) for the control (DW) and UT and BT samples. (b) SOD activity (U mg-1 FW) for DW, UT, and BT samples. Bacterial toxicity of DW, BT, and UT samples (c) Acute toxicity (d) Chronic toxicity.

4.3.13.3 Bacterial toxicity assay

Acute and chronic toxicity was measured after 15 min and 16 h, respectively, after inoculation of bioluminescent bacteria (*Photorhabdus luminescence subsp. Akhurstii)*. Bioluminescence intensity was assessed using HORIBA fluorescence spectrophotometer (PTI QM-400) in the range of 400-800 nm and noted as count per second (CPS). From **Fig. 4.15 (c)** and **(d)**, it is clearly visible that the bioluminescence intensity for the UT sample was reduced in

comparison to the BT sample. For chronic exposure % bioluminescence inhibition was 38.0 % for the BT sample and 81.5 % for UT sample.

4.4 Conclusion

In the present study, the biodegradation of BG dye was performed in PBBR using *Bacillus lichenifomis* immobilized PUF as packing material. The experimental data were fitted with the statistical models (i.e., RSM and ANN), and the higher values of coefficient determination ($R^2 >$ 0.99) show the accuracy of the prediction. In the case of highly non-linear processes like dye biodegradation, ANN may be a preferable option to RSM. PBBR showed excellent dye removal efficiency (RE) when operated under optimum conditions. Maximum RE of 91.2% and elimination capacity of 21.88 mg/L.d were obtained when PBBR was operated at a low inlet loading rate (24.0 mg/L.d). The biodegradation kinetics was analyzed using Monod and Andrews-Haldane models, and the substrate inhibition was observed beyond 170 mg/L of BG dye. The content of chlorophyll (a, b) was notably reduced in the untreated sample. SOD activity was noticeably elevated for untreated samples, demonstrating the significance of these enzymes in plant tolerance to dye contamination. The biologically treated sample showed less toxicity towards *P. luminescence* bacteria in comparison to the untreated sample. This biofilm-mediated system may further be extended to treat the wide range of pollutants from wastewater.