CHAPTER 3

Biodegradation and detoxification study of triphenylmethane dye (Brilliant Green) in a recirculating packed-bed bioreactor by bacterial consortium

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3.1 Introduction

Brilliant green (BG), a triphenylmethane- based cationic dye, has extensive applications in various industries such as leather, biological stain, varnishes, dermatological agent, plastics, and textiles (Khan et al., 2020). Direct exposure to BG dye causes gastrointestinal tract irritation with symptoms of diarrhea, nausea, respiratory tract irritation, shortness of breath, cough, and vomiting. The gradual accumulation of synthetic dyes and their counterpart molecules in the soil leads to the transfer of these molecules into groundwater through leaching, imposing serious threats to future generations (Vikrant et al., 2018). Therefore, the wastewater contaminated with textile dyes need an innovative, advanced, sustainable, economic, and environmentally friendly method to eliminate the adverse impacts.

Nowadays, Biological treatment (i.e., Bioremediation) emerges as a promising technological tool to treat the dyes containing wastewater. Microorganisms such as *Aeromonas hydrophila, Klebsiella quasipneumoniae, Pseudomonas aeruginosa, Aeromonas sp., Shewanella oneidensis,* and *Bacillus sp.*, etc. have shown promising results toward biodegradation of textile dyes (Bharti et al., 2019). The bioremediation technique deals with the activity and growth of microbes and their efficacy towards waste mineralization, either *in-situ* or *ex-situ*, under suitable environmental conditions.

In biodegradation, generally, two types of system, namely suspended growth (i.e., free cells) and attached growth (i.e., Immobilized cells) are used. The former employs the direct contact of microbes with the pollutant, whereas the latter uses packing support materials onto which microbial biofilm formed. The attached growth system shows advantages such as high specific surface area, high stability under adverse conditions, and biomass reuse over a suspended growth system (Banerjee and Ghoshal, 2017). According to Sonwani et al. (2020), an attached growth system exhibited better performance compared with free cell systems.

In the attached growth system, bioreactors, including the Membrane Bioreactor (MBR) (Kuppusamy et al., 2017), Fluidized Bed Bioreactor (FBBR), Moving Bed Biofilm Reactor (MBBR), Rotating Biological Contactor (RBC), and Packed Bed Bioreactor (PBBR) (Swathi et al., 2021) are widely employed. Among them, PBBR provides several process benefits such as process simplicity, fast reaction, high mass transfer rate, and product quality that can be easily controlled (Sen et al., 2017).

The present study investigated the biological degradation of brilliant green (BG) dye in PBBR under various conditions. A modified carrier, namely polypropylene-polyurethane foam (PP-PUF) immobilized with *Alcaligenes sp.* SY1 and *Enterobacter asburiae* strain SG43 were used in PBBR. The performance of a continuous recirculating packed bed bioreactor (RPBBR) was examined at various organic loading rates. Analytical tools such as UV-Vis Spectrophotometer, FTIR, and ^{1}H NMR were employed to analyze the samples. Based on previous research, a limited ^{1}H NMR study for the biodegradation of textile dyes was conducted. To the best of the authors' knowledge, this is the first study in which a ^{1}H NMR spectrum elucidated the structural transformation of BG dye after biodegradation.

3.2 Materials and methods

3.2.1 Dye and chemicals

Brilliant green (CAS number 633-03-4, $C_{27}H_{33}N_2HO_4S$, λ_{max} 630 nm, MW 482.64 g/mol), a commonly used triphenylmethane-based dye, was purchased from Sigma-Aldrich, India. The actual dye content (i.e., purity) was 90% and its solubility in the aqueous solution was 100 g/L at 20 °C. The mineral salt media (MSM) composition was as follows (g/L): K_2HPO_4: 1.73, KH_2PO_4: 0.63, $MgSO_4.7H_2O$: 0.1, NaCl: 4, $FeSO_4.7H_2O$: 0.03, NH_4NO_3:1, $CaCl_2.2H_2O$: 0.02, beef extract: 2.0, peptone: 1.0, and glucose: 1.5. Peptone and Glucose were the additional Nitrogen and Carbon supplementary nutrients for enhancing the activity of microbial consortium during immobilization period. All of the chemicals and microbiological media used in this study were of the highest purity and analytical grade, purchased from Merck, India. The synthetic wastewater used for biodegradation analysis was prepared in sterilized MSM with different concentrations of BG dye. The final pH of synthetic textile wastewater was adjusted with the addition of 0.1 N HCl/0.1 N NaOH. **Table 3.1** depicts the characteristics of simulated BG dye wastewater.

Table 3.1 BG dye simulated textile wastewater characteristics.

S.N.	Parameters	Concentration
1.	pH	7.0 ± 0.5
2.	Chemical oxygen demand (COD) (mg/L)	2750 ± 137.5
3.	Total suspended solid (TSS) (mg/L)	735 ± 36.75
4.	Total organic carbon (TOC) (mg/L)	570 ± 28.5
5.	Total alkalinity (mg/L)	285 ± 10

3.2.2 Isolation of dye degrading bacterial culture and molecular characterization

It has been well-known that the potential dye degrading bacterial culture is always present in nearby contaminated soil and wastewater of textile allied industries. Therefore, the soil sample was collected from the top 15 cm layer of soil near the textile waste contaminated industrial sites of Ambey Processors, Gida, Sahjanwa (28°29ʹ0.384ʺ N, 77°30ʹ29.2248ʺ E) U.P, India. The soil sample was carried out in sterilized polyethylene bags and stored at 4 °C for further use. The enrichment of microbial culture was accomplished by allowing in gradually increasing concentration (i.e., 50 to 200 mg/L) of BG dye for 7.0 days of incubation. Enrichment followed by isolation of bacterial species using serial dilution was performed. The isolated bacterial cultures were preserved in a NaCl-yeast extract (NY) medium having 4% NaCl and 0.5% yeast (Chaturvedi et al., 2021).

Microbial species identification, classification, and quantification are frequently accomplished using the sequencing of the 16S rRNA gene sequencing. 16S rRNA sequencing is commonly utilized in the identification of bacteria and phylogenetic research because the 16S rRNA gene is conserved in bacteria and contains hypervariable areas that can offer species-specific signature sequences. High precision, low cost, and quick sequencing of the 16S rRNA are its main advantages. The molecular characterization of isolated potential bacterial species was carried out in Bioraj Laboratories, Pune, India. The AxyPrep Bacterial Genomic DNA Miniprep kit was used to extract and purify genomic DNA from bacterial species, and Nanodrop was used to ensure purity and quantification. In the amplification, universal 16S rRNA PCR (polymerase chain reaction) forward primer (27F-5′ AGAGTTTGATCMTGGCTCAG3′) and reverse primer (1492R-5′ AAGGAGGTGWTCCARCC3′) were used (Sonwani et al., 2020). PCR reaction was performed in a thermal cycler (Bio-rad) using the following conditions: initial denaturation of 5 min at 94 °C, followed by 35 cycles consisting of 30 s at 94 °C (denaturation), 20 s at 58 °C (annealing), and 1 min 30 s at 72 °C (extension) and final extension was 15 min at 72 °C. The PCR products were analyzed by 1.2% agarose gel electrophoresis, amplified products in the gel were cut by a clean scalpel, purified by Quigen quick PCR purification kit and DNA was sequenced. The obtained 16S rRNA sequences were further subjected to the BLAST program (http://www.ncbi.nlm.nih.gov/) to confirm the organisms.

3.2.3 Packing material and experimental set-up

Polyurethane foam (PUF) (300 ×300 mm, 8 mm thickness) and Polypropylene (PP) sheets (39 x 78 inches, 10 mm thickness) were selected as a support bio-carrier for RPBBR. These materials were purchased from the local market, in Varanasi, India. PP-PUF bio-carriers were selected because they exhibited sufficient mechanical strength, high porosity, were economically cheap, and facilitate stable biofilm formation (Shalini and Y., 2019). The density and specific surface area of PUF were 80 kg/m^3 and 280 m^2/m^3, whereas 906 kg/m^3 and 530 m^2/m^3 for PP, respectively. The PUF foam cubes were cut into small pieces (2×2 cm) and fixed into alternate holes of PP. The immobilization and formation of biofilm had achieved using a modified Polypropylene-Polyurethane foam (PP-PUF) carrier (each having an average weight of 1.264 ± 0.06 g). The contact angle of the PP-PUF bio-carrier was 64.32° with water and ensuring its hydrophilic nature.

The microorganisms were subjected to growth over the packing material before beginning the bioreactor study. The required nutrient medium was supplemented during the bacterial cell acclimatization period (15 days). The SEM studies revealed the formation of stable biofilm on the packing support material. As the bacterial species begin to grow they secrete extracellular polymeric substances (EPS), which create a protective matrix around the biofilm. EPS enhances biofilm cohesion and stability. The dye wastewater was fed with relatively low-to-moderate volumetric flow rates. The stable biofilm resists the detachment or disruption as the pressure drop increases. The energy consumption during the process is due to the continuous operation of the peristaltic pump. However, the energy consumption by the pump is not a significant parameter, thus the research studies do not critically highlight it. Since the major attention was laid on the efficient degradation and detoxification of the dye wastewater.

In the present study, the experimental set-up was consisted of a feed tank, rotameters (SRS-MG5, Eureka, Pune), RPBBR bioreactors, and an air compressor (KNF, Labport, Germany), and peristaltic pumps (ELECTROLAB, PP- 50V) (**Figure 3.1**). Indigenously designed laboratory-scale up-flow RPBBR was fabricated with borosilicate glass material (1000 mm length, 50 mm diameter, and 2.5 mm thickness) with 1.0 L of working capacity. The air was fed by an air compressor, and passed through a 0.44 µm filter followed by an air sparger at the bottom port. The flow rate was controlled by the rotameter.

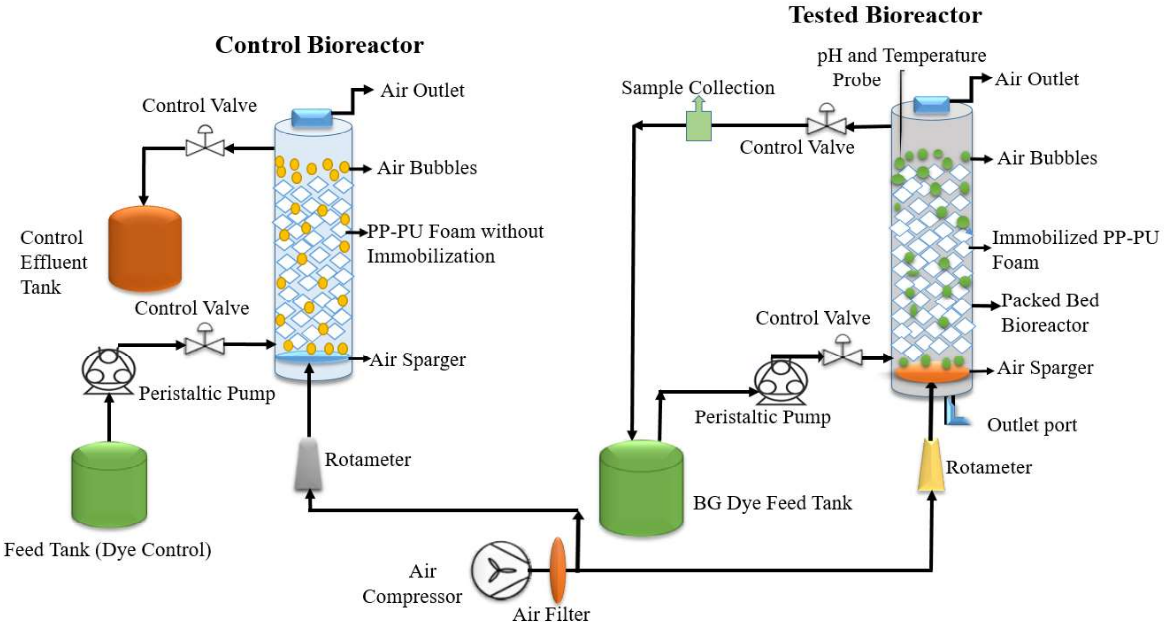


Figure 3.1. Schematic representation of the indigenously developed laboratory scale recirculating packed bed bioreactor (RPBBR)

A bioreactor was filled with PP-PUF bio-carriers followed by adding isolated microbes as inoculum (Swain et al., 2020). A control bioreactor was filled with PP-PUF bio-carriers without microbes. It was operated simultaneously with an RPBBR as control. The bioreactor system was allowed to operate continuously for 20 days of operation. Throughout the bioreactor operation, the physicochemical parameters (e.g., COD, TOC, and TSS) and dye biodegradation were periodically monitored at different organic loading rates (OLR) (i.e., 2.7, 1.27, 0.93, 0.71, and 0.53 kg COD/m^3 .d^{-1} of BG dye influents) and HRT. OLR of BG dye was allowed to vary at different time intervals during the bioreactor operation. The reactor was operated at room temperature of 27 ± 3 °C and adjusted pH of 7 ± 0.5. The dye wastewater influent was fed at the bottom to eliminate the channeling effect and increase the retention time. The working volume of the reactor was 800 mL out of 1000 mL total volume. The isolated bacterial cultures were inoculated into the RPBBR until biofilm formed within the pores and surface of the bio-carrier. The BG dye wastewater was fed to the reactor via the peristaltic pump at the flow rate of 120 ± 2 mL/h. Dissolved oxygen (DO) was maintained throughout the experiment at 5 ± 2 mg/L. All the samples were withdrawn at a regular time interval and biodegradation studies were carried out in a triplicate manner.

3.2.4 Analytical approaches for quantifying dye mineralization

The treated effluent was collected, centrifuged at 5000 rpm for 10 min (REMI CENTRIFUGE, RM- 12C BL), filtered with a 0.22 μm filter, and the cell-free clear supernatant had examined for dye degradation performance. The extent of dye mineralization was assessed in terms of reduction in COD, TSS, and TOC. The COD and TSS were determined using the standard protocol APHA (1998). The TOC of the sample was determined by the TOC analyzer (multi N/C 2100/2100S, Analytikjena, Germany). The absorbance of the biodegraded sample was measured at 630 nm by UV-Vis spectrophotometer (SL-159, Elico, India). The standard curve of dye was withdrawn at room temperature, and dye degradation was calculated by using the following equation:

$$\text{Dye degradation (\%)} = \frac{\mathrm{OD_1 - OD_t}}{\mathrm{OD_1}} \times 100 \tag{3.1}$$

where OD_1 and OD_t refer to the initial absorbance before degradation and absorbance after degradation respectively.

3.2.5 Characterizations

BG dye biodegraded intermediates/metabolites were extracted using an equal volume of ethyl acetate, dried over anhydrous sodium sulfate followed by concentrated into a rotary vacuum evaporator. The obtained crystals were dissolved with HPLC grade methanol and further analyzed spectroscopically. Fourier Transform Infrared Spectroscopy (FTIR) analysis of control dye and extracted metabolites was carried out for the confirmation of changes in the functional groups. It is a powerful analytical technique used to study the interactions between molecules based on their absorption and emission of infrared radiation. By analyzing the characteristic vibrations of functional groups within a sample, FTIR provides valuable insights into molecular composition, chemical bonds, and structural arrangements. The spectra were recorded by the FTIR spectrophotometer (Nicolet iS5, THERMO) in the mid-infrared region of 400-4000 cm^{-1} with 32 scan speed and 4 cm^{-1} resolutions. The sample was pre-mixed with spectroscopically pure KBr (Potassium bromide) in a ratio of 5:95. The spectrum analysis was performed in a triplicate manner and necessary baseline correction was done to predict the change in the structure of BG dye. Furthermore, Proton Nuclear Magnetic Resonance (^1H NMR) spectrometry analysis of control dye and the extracted metabolites were analyzed by NMR Spectrometer (AVANCE III HD 500 MHz, BRUKER). It is a sophisticated analytical technique that examines the behavior of hydrogen nuclei within a molecule when placed in a magnetic field. This method reveals vital structural information about compounds, identifying chemical structures, functional groups, and molecular arrangements. ^1H NMR spectra observed the structural transformation in dye molecule during biodegradation, incorporating tetramethylsilane (TMS) as standard. The dried samples were dissolved in Deuterated chloroform ($CDCl_3$) and transferred to 5 mm diameter NMR tubes (Ayed et al., 2017; Pandey et al., 2020). Dissolved oxygen (DO) level (HD 2109.1; Delta OHM; Italy) and pH (LMPH-10; LABMAN; L8119) of the dye effluents were measured periodically during the bioreactor operation. The morphological analysis of the bio-carrier and after the biofilm formation on the bio-carriers were carried out using Scanning Electron Microscope (SEM) (QUANTA 450).

3.2.6 Phytotoxicity assessment of biodegraded dye effluents

Toxicity analysis is embedded with the performance of the bioreactor. Phytotoxicity refers to the harmful effects that certain substances, such as chemicals or pollutants, can have on plants. It involves the study of how these substances can interfere with the growth, development, and overall health of plants. Phytotoxicity assessments are crucial to understanding the potential impact of various compounds on agricultural crops, natural vegetation, and the environment. Seed germination is one of the easy and popular methods for conducting *in-situ* phytoremediation (Sutar et al., 2019). The analysis was conducted using *Phaseolus mungo* seeds to assess the toxicity of the biodegraded and control samples. Before sowing, seeds were sterilized with 0.1% $HgCl_2$ and washed several times with distilled water. The biomass-free supernatants (2 mL) of the treated and untreated samples were added to the 10 seeds (in soil) and were incubated under controlled conditions (Temperature of 30 ± 5 °C and relative humidity of 50 ± 5%). To maintain the moisture content, 5 mL of distilled water was added every 24 h of intervals. The supernatant was replaced with distilled water in the case of a control sample. The experiment was performed with 250 mg/L of BG dye. After the 7th day of incubation, the toxicity assessment was articulated in terms of seed germination (%) and length of plumule (shoot) and radical (root). The *Phaseolus mungo* seed germination percentage was calculated by the following formula:

$$\text{Seed germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Total seeds sowed}} \times 100 \tag{3.2}$$

3.3 Results and discussion

3.3.1 Identification of dye degrading bacterial species

Initially, five different bacterial species (i.e., BGT1, BGT2, BGT3, BGT4, and BGT5) were isolated from the soil sample. These bacterial species were tested for the biodegradation of BG dye (100 mg/L). Bacterial growth was measured at 600 nm. The bacterial species, namely BGT1 and BGT2 showed a better growth rate than other species (**Figure 3.2**). Therefore, BGT1 and BGT2 bacterial species were analyzed by 16S rRNA sequencing. RNA sequencing revealed that BGT1 and BGT2 were *Enterobacter asburiae* strain SG43 and *Alcaligenes* sp. SY1, respectively. The sequences were deposited in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and obtained the accession numbers KR856388 and KP162176 for BGT1 and BGT2, respectively. The phylogenetic tree of both the bacterial species are illustrated in **Figure 3.3**.

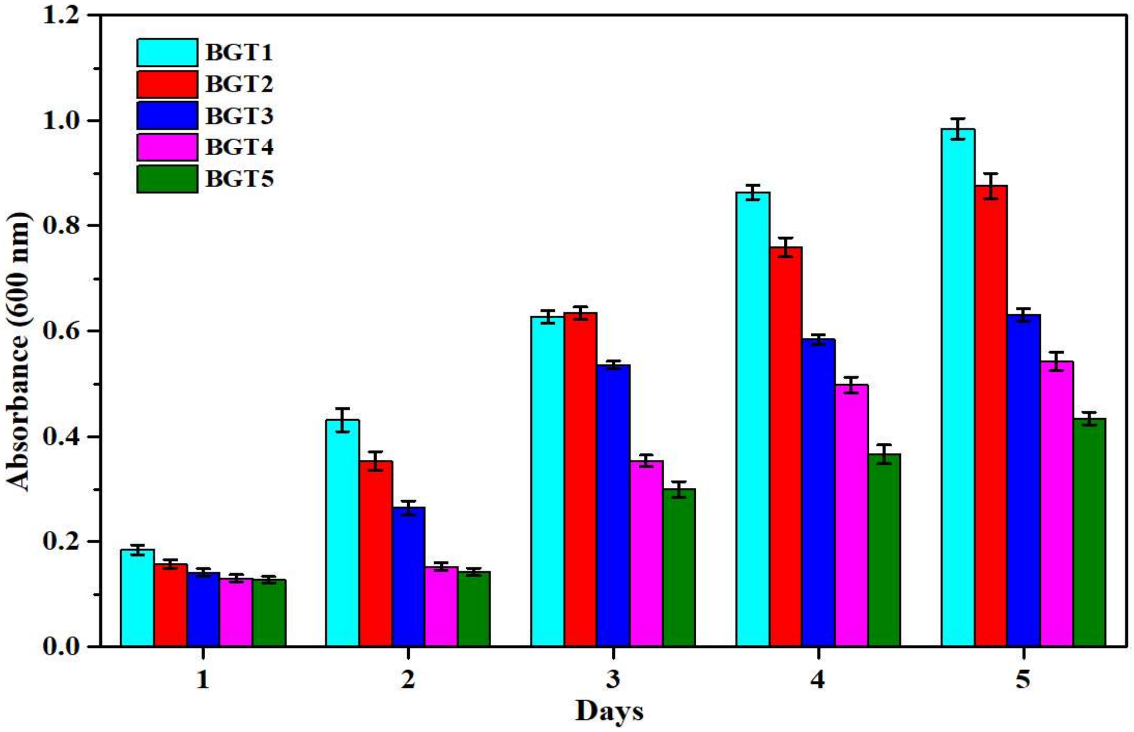
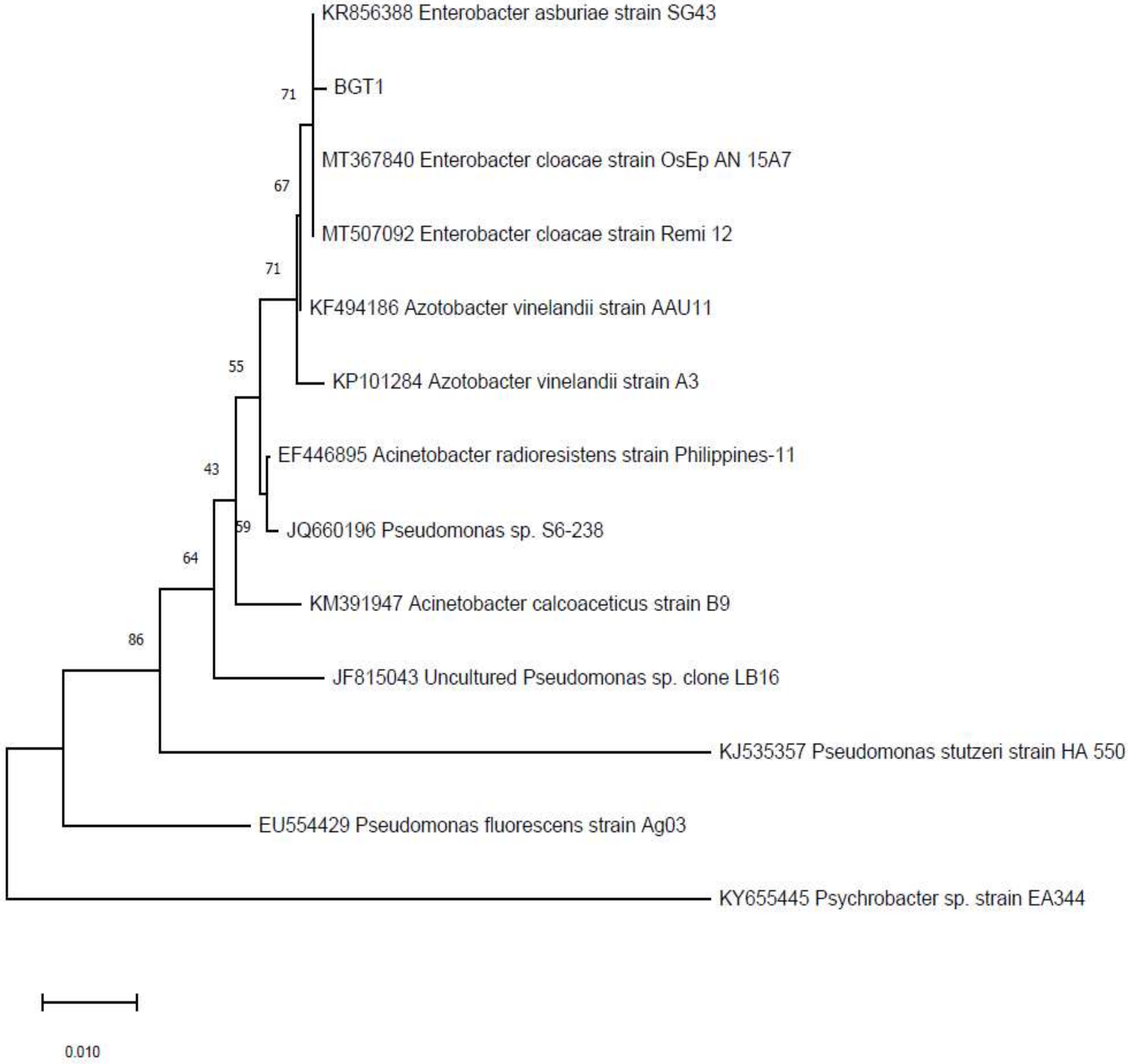
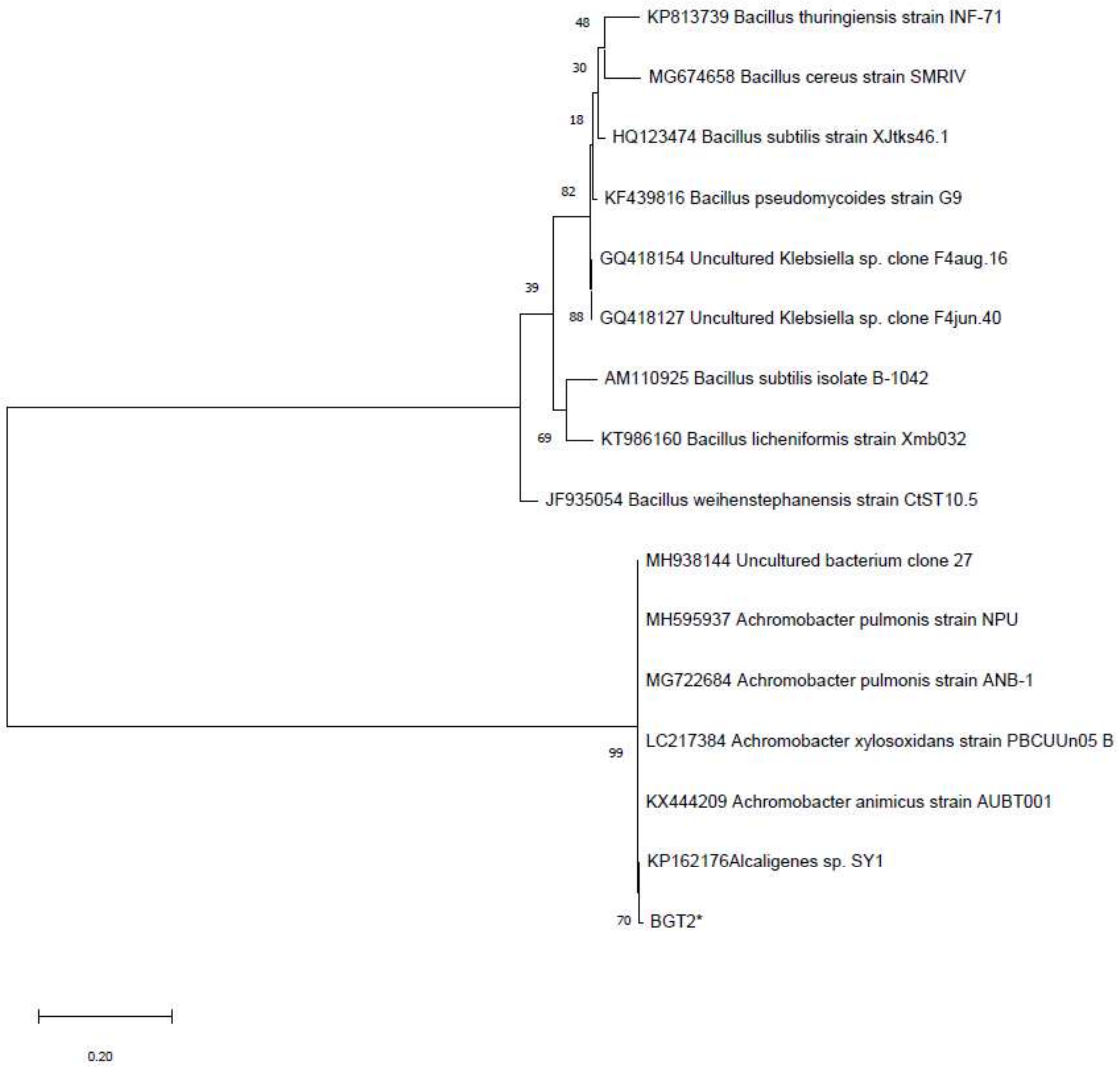


Figure 3.2. Growth of bacterial species in BG dye under 100 mg/L of initial concentration (Error bars depict the standard deviation)


KR856388 Enterobacter asburiae strain SG43
BGT1
MT367840 Enterobacter cloacae strain OsEp AN 15A7
MT507092 Enterobacter cloacae strain Remi 12
KF494186 Azotobacter vinelandii strain AAU11
KP101284 Azotobacter vinelandii strain A3
EF446895 Acinetobacter radioresistens strain Philippines-11
JQ660196 Pseudomonas sp. S6-238
KM391947 Acinetobacter calcoaceticus strain B9
JF815043 Uncultured Pseudomonas sp. clone LB16
KJ535357 Pseudomonas stutzeri strain HA 550
EU554429 Pseudomonas fluorescens strain Ag03
KY655445 Psychrobacter sp. strain EA344
71
67
71
55
43
59
64
86
0.010

(a)



(b)

Figure 3.3. Phylogenetic tree of isolated bacterial species (a) *Enterobacter asburiae* strain SG43, and (b) *Alcaligenes* sp. SY1

3.3.2 Biodegradation study of Brilliant green dye in a continuous bioreactor

One of the critical factors governing the performance of the biodegradation process is the stability of PBBR at different OLRs. To assess biodegradation performance, the RPBBR was subjected to varying inlet OLRs. The extent of mineralization (TOC, COD, and TSS removal) and dye degradation at varying OLRs were measured for 20 days (triplicate study) of bioreactor operation, as shown in **Figure 3.4**. The degradation of BG dye took place due to the synergistic action of bacterial consortium. Oxygen served as the aerobic environment for the survival of the bacterial species. Because the dye OLR was varied during the bioreactor study, it could be a viable approach for the growth promotion of the aerobic bacterial species. Since the solubility of oxygen was low and its depletion occurred during the biodegradation process. Therefore, aeration could somehow maintain the necessary oxygen concentration throughout the biodegradation process. The degradation process began due to the bacterial action not due to the oxidation through aeration. The bioreactor was initially fed with 2700 mg/L of COD and a dye concentration of 1000 mg/L. The initial TOC and TSS of BG simulated dyeing wastewater was 570 ± 28.5 and 735 ± 36.75 mg/L, respectively. After 4 days, the COD, TOC, TSS, and BG dye concentrations were reached 1535 ± 76.75 mg/L, 436 ± 21.8 mg/L, 632 ± 31.6 mg/L, and 760 ± 38 mg/L, respectively. The bioreactor was subsequently operated on with an OLR of 1.27 kg COD/m^3 .d^{-1} (COD = 1270 mg/L) and an HRT of 4 days. Further, the bioreactor reduced the dye concentration, TOC, COD, and TSS to 500 ± 25 mg/L, 292 ± 14.6, 984 ± 49.2, and 526 ± 26.3 mg/L, respectively after 8 days of operation. Thus, after an HRT of 8 days, the bioreactor achieved 50% dye reduction, 64.2% COD, 48.7% TOC, and 28.4% TSS reduction at steady-state. Now, the HRT was allowed to increase process time from 8 days to 12 days and inlet OLR was maintained at 0.93 kg COD/m^3 .d^{-1}. As the OLR decreased and HRT increased, the biodegradation performance increased remarkably. At the end of the 12th day of bioreactor operation, the dye concentration was 320 ± 16 mg/L, whereas COD, TOC, and TSS concentrations were reached at 765 ± 38.25, 172 ± 8.6, and 456 ± 22.8 mg/L, respectively. For OLRs of 0.71 and 0.53 kg COD/m^3 .d^{-1}, the inlet OLR was fine-tuned again, and the bioreactor process performance was evaluated. The bioreactor reached its final steady-state after the 20th day. The BG dye concentration was 90 ± 4.5 mg/L, while the final steady-state values for COD, TOC, and TSS were 385 ± 19.25, 82 ± 4.1, and 366 ±18.3 mg/L, respectively. On the 20th day, the removals of BG dye, COD, TOC, and TSS were 91%, 86%, 85.6%, and 50.2%, respectively.

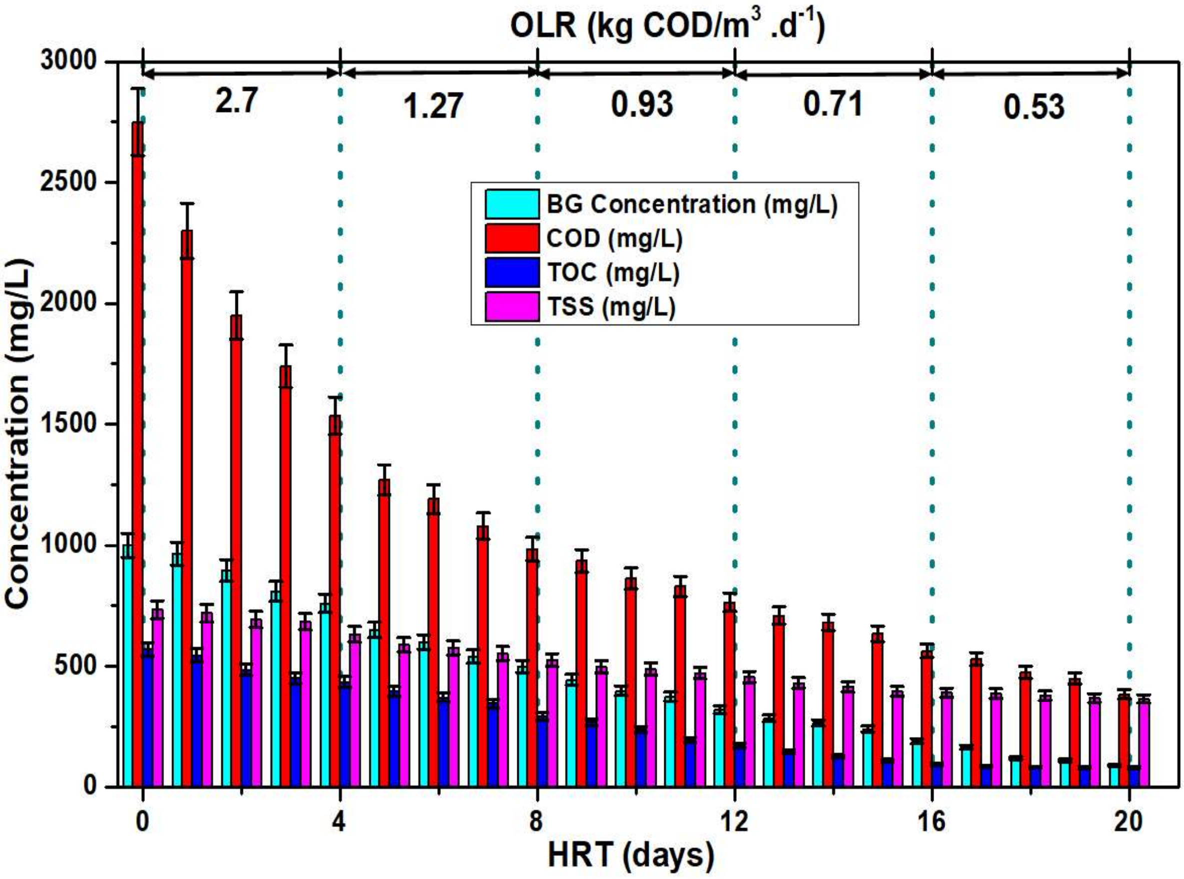


Figure 3.4. Performance evaluation of a continuous RPBBR. Effect of organic loading rate (OLR) on the COD, TOC, TSS reduction, and biodegradation of BG dye (Error bars depict the standard deviation of triplicate experiments)

Various researchers have conducted similar studies to investigate the impact of OLRs and HRT in the biodegradation of dyes (Balapure et al., 2015; Rajasimman et al., 2017; Rathour et al., 2021). Rathour et al. (2021) recently conducted a study on the biodegradation of raw textile effluents (RTE) in an integrated upflow fixed-film microaerophilic-aerobic bioreactor (UFMB-AB) system. They investigated the effect of OLR (from 7.54 to 0.83 kg COD/m^3 .d^{-1}) and HRT (5 days) on bioreactor performance. The study found that the integrated system reduced COD from 8380 ± 78 mg/L to <50 mg/L, TSS from 1100 ± 60 to 334 ± 20 mg/L, and color from

2171 ± 18 to 177.38 ± 13.47 mg/L. The bioreactor performance is substantially affected by a higher inlet OLR. This could be owing to the presence of a larger concentration of dyes, which inhibits the enzymatic activity of the bacterial consortium, lowering the performance of the bioreactor (Bankole et al., 2018; Yadav et al., 2014).

Table 3.2 Comparison of Brilliant green dye biodegradation study with the available literature.

Dye	Bioreactor	Microorganism	Dye concentration (mg/L)	Degradation efficiency (%)	Reference
Evans blue and brilliant green dye mixture	Fed-batch bioreactors	*Citrobacter* sp., *Aeromonas hydrophila* and *Pseudomonas putida*	170–200	95.2–100% and 84.0–98.2%	(Zabłocka-Godlewska and Przystaś, 2020)
Brilliant green	Batch study	*Aspergillus* sp. strain CB-TKL-1	70	99.27%	(Kumar et al., 2012)
Brilliant green	Co-culture technology	*Pleurotus florida* and *Rhizoctonia solani*	20000 2% (w/v)	98.54%	(Kumari and Naraian, 2016)
Brilliant green	Bio-sorption	*Lenzites elegans* WDP2	-	92.77%,	(Pandey et al., 2018)
Brilliant green	Alginate beads-packed bioreactor	*Bjerkandera adusta* SM46	500	10.8–97.3%	(Andriani and Yanto, 2021)
Brilliant green	Batch study	*Klebsiella* strains	100	81.4%	(Zablocka-Godlewska et al., 2015)
Brilliant green	Recirculating Packed bed bioreactor (RPBBR)	*Alcaligenes sp. SY1 and Enterobacter asburiae strain SG43*	1000	91%	**Present study**

3.3.3 Biodegradation analysis

3.3.3.1 UV-Vis spectrophotometer analysis

The BG dye biodegradation analysis was confirmed through the comparative analysis of the UV-vis absorbance spectral profile of the control sample (200 mg/L) and biodegraded sample (**Figure 3.5**). There was a significant decrease in the absorbance value of the biodegraded sample at 630 nm, which confirmed the biodegradation of BG dye in RPBBR. The maximum spectral peak of BG control dye disappeared almost completely after biodegradation. The residual absorbance value of the biodegraded dye solution suggested that the complete mineralization could not be achieved in the biodegradation of dye.

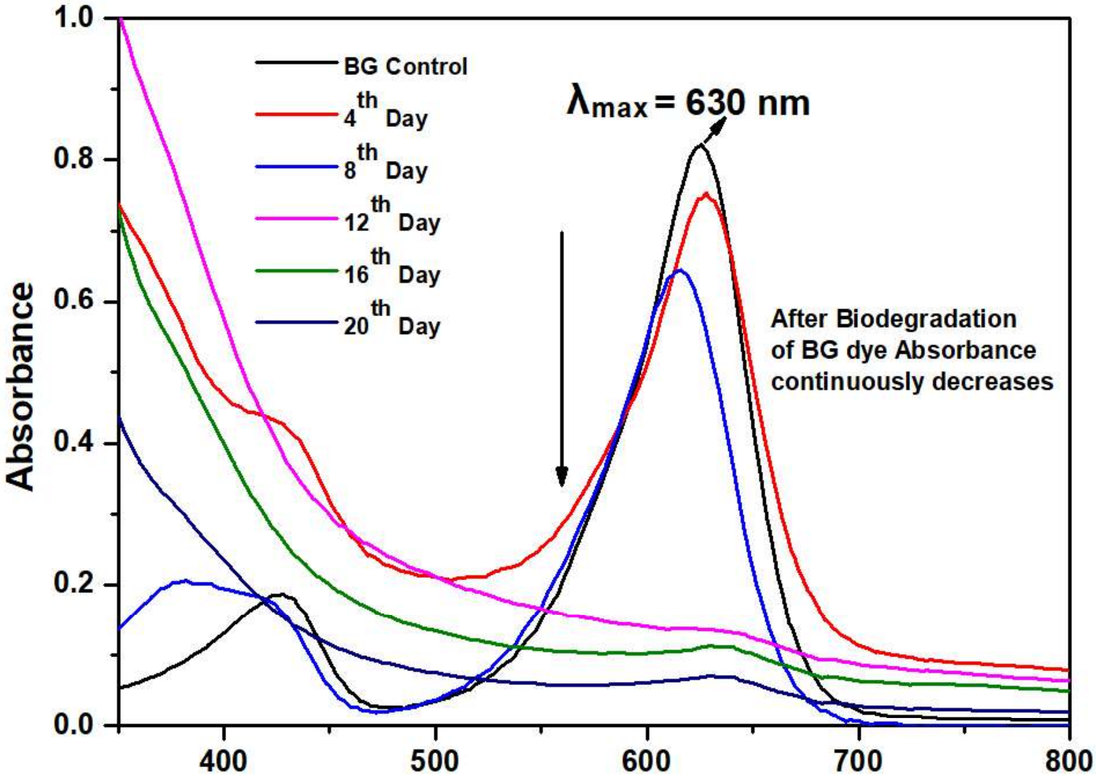


Figure 3.5. UV-Vis absorbance spectral profile of BG dye and its biodegraded sample in RPBBR during various intermediate durations (4th, 12th, 16th, and 20th day)

3.3.3.2 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectra

A comparative study of ^{1}H NMR spectra of initial (control) dye and its biodegraded solution is reasonably able to depict the biodegradation process. ^{1}H NMR spectra of control dye solution showed major downshift multiple signals between chemical shift (δ) 7.285, δ 7.692, δ 6.973, and δ 6.991, which were attributed to the protons of three aromatic rings present in the triphenylmethane BG dye (**Figure 3.6a**). The minor signals between δ 3.681 to δ 3.724 confirmed the presence of proton of R_2 N-H (amine) groups. The single singlet at δ 2.112 was due to R=C-H (alkene) group, whereas the presence of saturated alkanes was confirmed by the signals between high field zone δ 1.252 to δ 1.383. The biodegraded solution of BG dye exhibited a small signal at δ 7.281 and there was no major signal appeared in the aromatics region (**Figure 3.6b**). The complete elimination of aromatics signals significantly suggested that BG dye was biodegraded by the bacterial culture of *Enterobacter asburiae* strain SG43 and *Alcaligenes* sp. SY1. On the other hand, the major signal at δ 3.089 was attributed to the aromatic ring, namely the benzene ring (Ar-CH-R_2) and alkene (R=C-H). Other signals were obtained at δ 4.454 and δ 4.071, which suggested the presence of protons of R-NH (amine) groups. The spectrum of biodegraded solution showed the disappearance of major signal (δ 7.3 and δ 2.1) and formed a new signal (δ 3.089 and δ 4.454). The major aromatic signal had lost and formed only a single peak attributed to the finding that the protons present in the aromatic rings of BG dye were biodegraded and converted into the aliphatic compounds. This might be due to the opening of aromatic structures into the lower molecular weight aliphatic hydrocarbons. Thus the comparative study suggested that the entire BG dye molecule was biodegraded into aliphatic hydrocarbons, aliphatic amines, and alkenes. On the other hand, the aeration leads only to maintaining the growth environment so that the aerobic bacterial species do not lose their activity.

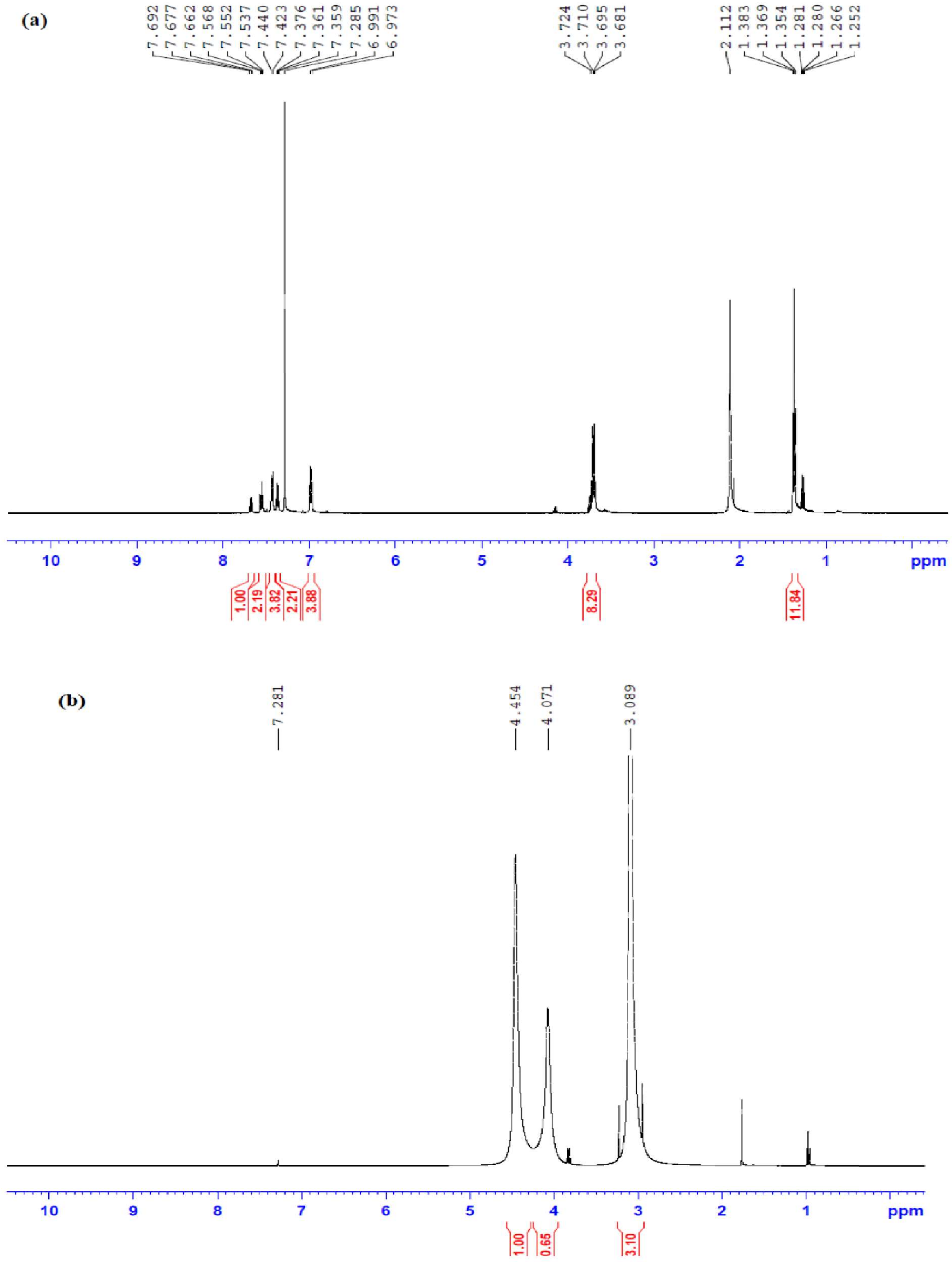


Figure 3.6. A typical comparison of ^{1}H NMR spectra (a) Brilliant green dye control and (b) After biodegradation

3.3.3.3 FTIR Spectral Analysis

The FTIR spectrum of untreated (control) dye and biodegraded dye solution is illustrated in **Figure 3.7**. FTIR spectrum of the control BG dye sample exhibited various peaks at different wavenumber. The peak at 615.5 cm^{-1} was due to the C-H vibration and bending of alkene, a peak at 799.16 cm^{-1} was due to the N-H strong broad stretching of amine. The peaks at 1011.28, 1104.91, 1190.49, and 1254.13 cm^{-1} suggested the presence of medium C-N stretching of an aliphatic amine, the peak at 1368.96 cm^{-1} was attributed to C-H bending of alkane. Also, the strong C-C stretching of aromatics and medium C-C stretching of saturated alkene were exhibited at 1513.79 and 1611.81 cm^{-1}, respectively. An aliphatic C-H stretching of alkane was attributed at 2849.9 cm^{-1}, whereas the presence of secondary amine was shown at 2918.18 cm^{-1} wavenumbers. The broad peak at 3433.12 cm^{-1} was attributed to aliphatic amine N-H stretching. Moreover, the peaks between 1650 cm^{-1} to 2000 cm^{-1} had attributed to the presence of weak C-H bending of aromatic compounds. Biodegraded BG dye sample showed peaks at different wavenumbers. The sharp peak at 619.9 cm^{-1} was attributed to the strong and broad bending of alkene C-H, a peak at 990.07 cm^{-1} due to the monosubstituted strong C=C bending of alkene. The peaks at 1109.29 and 1177.32 cm^{-1} wavenumbers might be the presence of C-O stretching of aliphatic alcohols and/or carboxylic acids and also attributed to medium C-H stretching of amine. Similarly, a peak at 1394.57 cm^{-1} was due to medium C-H bending. A less broad peak appeared at 1637.4 cm^{-1} and showed C=N stretching, medium N-H stretching of primary amine was exhibited by the wavenumber 3466.7 cm^{-1}, respectively. A comparative study elucidated the disappearance and shifting of peaks, and new peaks appeared at 1394.57, and 1637.4 cm^{-1}. There were no aromatic stretching/ vibrations that appeared after the biodegradation. All the peaks obtained after the biodegradation were significantly in the favour of aromatic ring cleavage and thus formed aliphatic compounds. The FTIR spectra illustrated the key difference in the control and biodegraded sample of BG dye, which supports the biodegradation of BG dye in RPBBR.

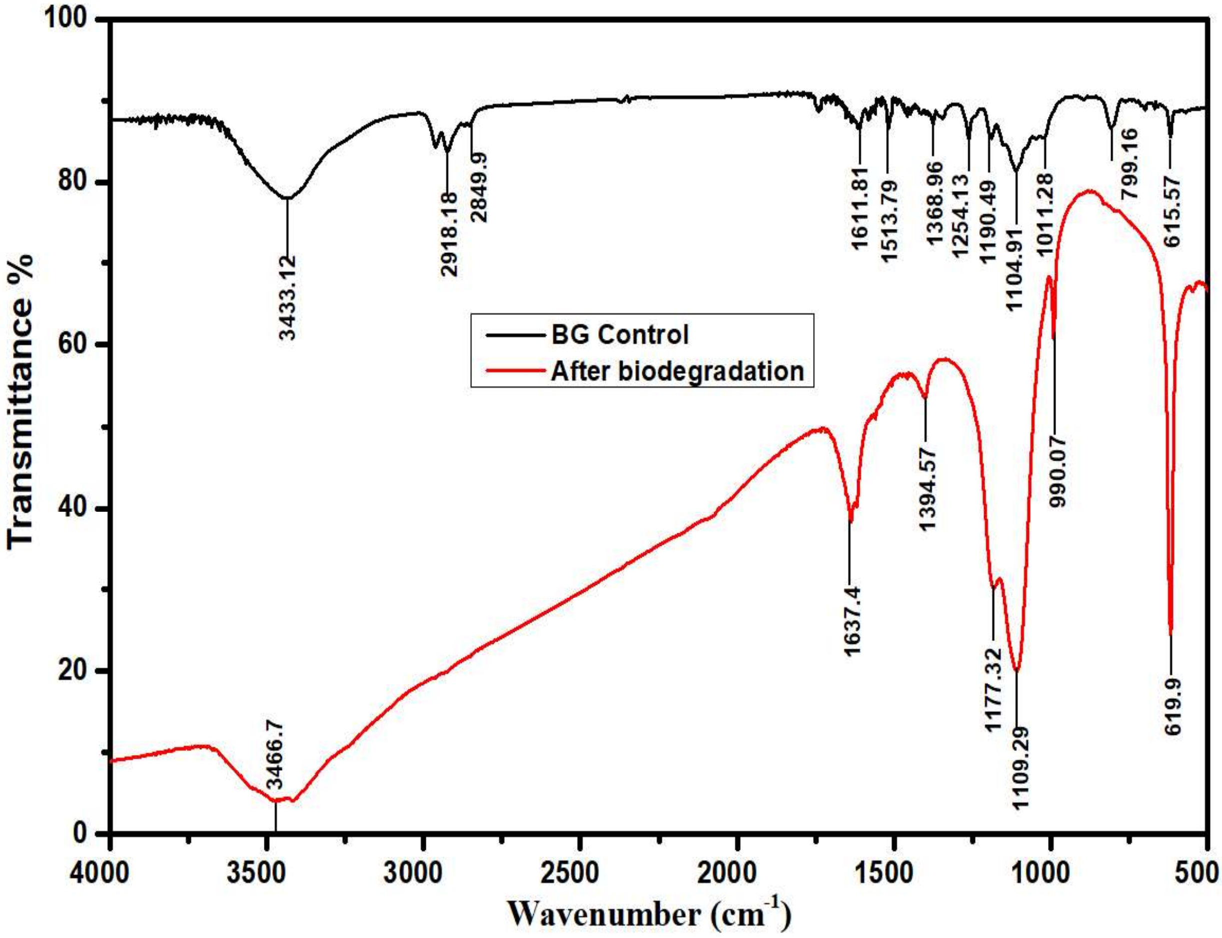


Figure 3.7. Typical FTIR spectra of BG dye control solution and biodegraded solution

3.3.4 Scanning Electron Microscope (SEM) Analysis

Polypropylene- Polyurethane foam was popularly employed for the promotion of the biofilm support material. SEM analysis of PP-PUF bio-carriers and after the biofilm formation was carried out (**Figure 3.8**). The morphology of PP-PUF bio-carriers clearly showed that the inner region was porous and facilitated the bacterial consortium to grow and form biofilm within the pores.

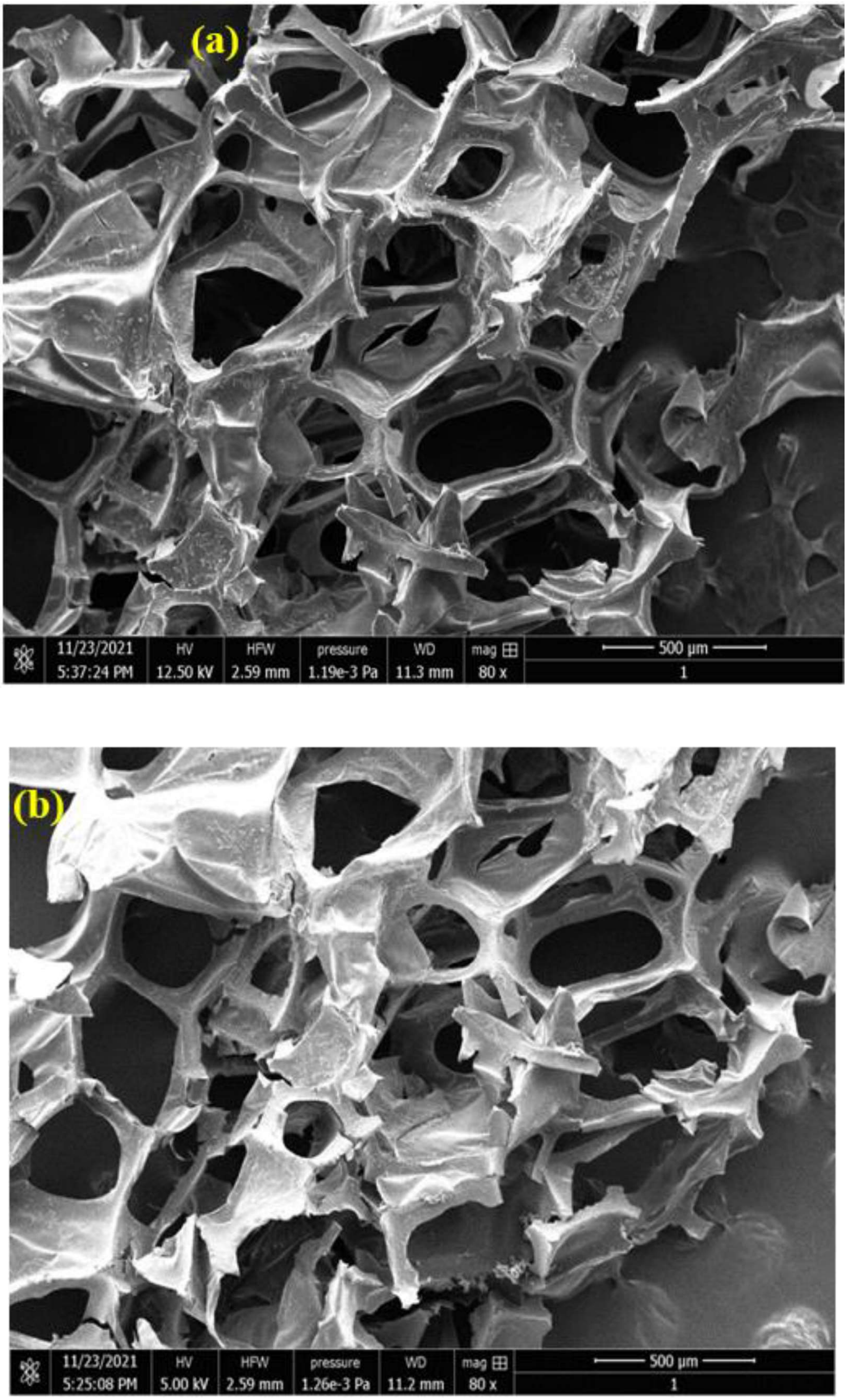


Figure 3.8. SEM images carrier (a) without immobilization (b) with immobilization

3.3.5 Phytotoxicity analysis

The direct exposure of toxic textile dye effluents into the environment expeditiously leads to severe hazards to aquatic life as well as to the plant species, diminishing the fertility of the soil. Quantitatively, phytotoxicity assessment of BG dye treated (i.e., biodegraded) and untreated (i.e., control) wastewater samples were expressed as the germination percentage of *Phaseolus mungo* seeds. *Phaseolus mungo* seeds were allowed to germinate in untreated and treated wastewater for 7.0 days. Due to the higher toxicity of BG dye in untreated wastewater, the seeds were able to exhibit only 32.4% germination (**Table 3.3**). However, the treated wastewater was relatively less toxic and achieved 83.56% germination. Moreover, the radicle/root and plumule/shoot lengths were found to be 1.3 ± 0.6 and 2.7 ± 0.4 cm, respectively for the simulated BG dyeing wastewater. Treated wastewater exhibited 5.6 ± 0.7 cm of root and 9.6 ± 0.8 cm of shoot lengths, respectively. Meanwhile, there was a remarkable improvement in the root and shoot lengths as well as in the germination rate of BG biodegraded dyeing effluents. Thus, this finding elucidated that BG biodegraded dyeing effluents toxicity was diminished through the bioremediation technique. A similar kind of phytotoxicity analysis was carried out for the electrochemically decolorized methyl red dye using *Vigna radiate* seeds achieved 80% germination, and the biodegraded solution exhibited 100% germination (Sathishkumar et al., 2017). The present findings of phytotoxicity results were very close to the previous findings that the toxicity of Methyl Red (Maniyam et al., 2018) and Reactive Red-195 (Saha et al., 2022) dyes was precisely reduced after the biodegradation. Thus, the above study elucidated the suitability of *Enterobacter asburiae* strain SG43 and *Alcaligenes* sp. SY1 towards the elimination of phytotoxicity of Brilliant Green in aqueous solution.

Table 3.3. Phytotoxicity assessment of BG dye and its biodegradation products.

Sample	Germination %	Root length (cm)	Shoot length (cm)
Distilled water	100 ± 0	8.5 ± 2.8	12.4 ± 3.9
Brilliant green dye control	32.4 ± 4	1.3 ± 0.6	2.7 ± 0.4
Treated effluents	83.56 ± 3.5	5.6 ± 0.7	9.6 ± 0.8

3.4 Conclusions

The present study incorporates the suitability of a typical RPBBR system for Brilliant green dye biodegradation. Isolated bacterial consortiums were found stress-tolerant against varying OLRs throughout the HRT of 20 days. The performance of RPBBR was evaluated at various OLRs. The maximum removal efficiencies of TOC, COD, BG dye, and TSS were obtained to be 85.6%, 86%, 91%, and 50.2%, respectively in RPBBR. Triphenylmethane-based BG dye has lost its aromaticity during the biodegradation, which is significantly depicted by ^{1}H NMR spectra. A comparative FTIR spectral analysis also ensures that the dye molecule after biodegradation is converted into the aliphatic hydrocarbons. Phytotoxicity assessment ensures the reusability of biodegraded effluents for irrigation. Thus, the incorporation of a packed bed bioreactor for the biodegradation of BG dye seems an ultimate approach.