CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

3.1. Equipments, materials, and glassware

The equipments were used in the present study such as incubator (NSW Ltd., India), laminar flow (Webcon Instruments Pvt. Ltd., India), autoclave, HPLC (UFLC Shimadzu, Japan), Gas chromatography-mass spectrophotometer (Shimadzu QP-2010), Scanning electron microscope (QUANTA 200F, Netherland), oven (NSW, Pvt. Ltd., India), centrifuge (REMI, RM-12C BL), chemical oxygen demand (UNIPHOS COD digester), dissolved oxygen (DO) meter (HD 2109.1; Delta OHM; Italy), pH meter (HD 2305.0; Delta OHM; Italy), UV-Visible spectrophotometer (ELICO, SL-210)), peristaltic pump (Milton Roy, India, A-11 SS), and sonicator (Labman Scinitific Instruments, India).

The materials like micro-pipette, silicon tube, cotton, inoculating loop, parafilm were brought from Samar enterprises, Varanasi, India. The Erlenmeyer flasks, volumetric cylinders, round bottom flasks, glass test tubes, centrifuge tubes, desiccators, L-shaped glass rod, petriplates, serum bottles were used after properly washing with double distilled water.

3.2. Chemicals and reagents

All the reagents used in the experiment were analytical grade. 4-chlorophenol and phenol were purchased from Himedia, India. HPLC grade chemicals (water and methanol) were purchased from Thermo Fisher Scientific Pvt. Ltd, India. All the chemicals used for the preparation of minimal salt medium (MSM) were brought from Merk, India. The wastewater was prepared by adding different amounts of phenol or 4-chlorophenol in MSM. The composition of MSM was summarized in Table 3.1 (Agarry and Aremu, 2012).

Table 3.1. Composition of MSM used for the preparation of wastewater

S.N.	Chemicals	Amount (g/L)
1	KH_2PO_4	0.5
2	$NaHCO_3$	0.7
3	NaCl	0.5
4	$FeSO_4$	0.02
5	K_2HPO_4	1.0
6	$CaCl_2.2H_2O$	0.02
7	$CuSO_4.5H_2O$	0.02
8	$MnSO_4.H_2O$	0.02
9	H_3BO_3	0.01
10	$Na_2MoO_4.2H_2O$	0.02

3.3. Methods

3.3.1. Collection of soil sample and isolation of microbial species

The bacterial species were isolated from the petroleum-contaminated soil, which was collected from the IOCL refinery, Mathura, India (27º30'12" N, 77º40'19" E and 181 m elevation above sea level). The acclimatization and isolation procedure followed in this work has been represented in Figure 3.1. Initially, a 5.0 g soil sample was added in a conical flask (250 mL) containing 100 mL MSM and phenol (50 mg/L) to enrich the bacterial culture. This flask was incubated at 35 ºC and 120 rpm till the complete removal of phenol. The incubation period was repeated by transferring 20 mL of aliquot sample from the previous batch to a 250 mL flask containing 100 mL MSM with phenol of concentration 100 mg/L. Further, this

process was repeated four times by gradually increasing the phenol concentration for the enrichment of microbial culture in phenolic environments. The potential microbial species were obtained by the serial dilution method. A total of six (GS1-GS6) microbial strains were isolated. Finally, the individual strain was cultured in agar plates and nutrient broth to store at 4 °C for further use.

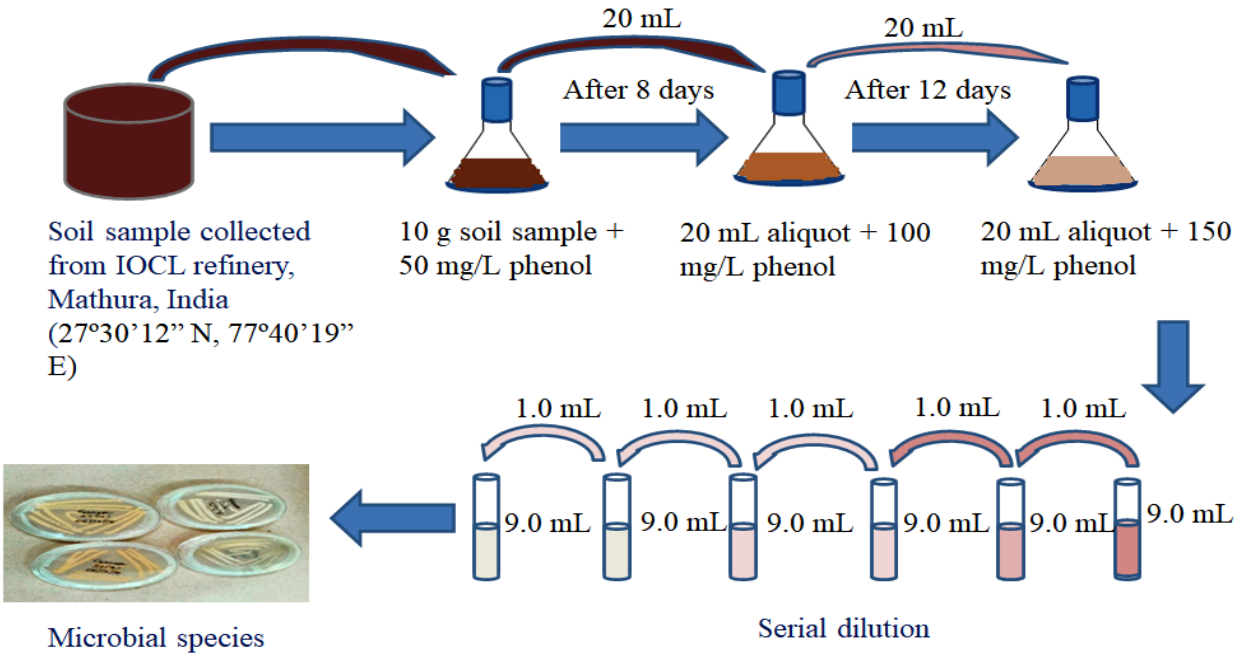

Figure 3.1. Process flow diagram of acclimatization and isolation of microbial species.

3.3.2. Selection of potential microbial species for the biodegradation of phenol

The preliminary batch study was carried out to evaluate the efficacy of individual microbial species for phenol removal. The free cell experiments were carried out at an initial phenol concentration of 100 mg/L (pH of 7.0, temperature of 35 °C). The bacterial inoculum (2.0 mL) was added to each Erlenmeyer flasks containing autoclaved MSM and phenol. A control was prepared in order to evaluate the abiotic losses of phenol. The flasks were incubated in an orbital shaker at 35 °C at 120 rpm for 10 days. The samples were collected on

a daily basis and anlysed in a HPLC for the determination of residual phenol concentration. The removal efficiency of phenol was calculated by using the following equation:

$$\text{Removal efficiency (\%) (RE)} = \frac{C_{in} - C_{out}}{C_{in}} \times 100 \quad (1)$$

where C_{in} (mg/L) and C_{out} (mg/L) correspond to the initial and final concentrations of phenol, respectively.

3.3.3. Identification of microbial species

The characterization of the potential microorganisms was carried out at Triyat Scientific, Nagpur, India. The DNA isolation and purification were done using the standard protocol (Sonwani et al., 2020). The DNA amplification was carried out by polymerase chain reaction (PCR) method using primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3'). The polymerase chain reaction (PCR) was used to make multiple copies of the DNA segment. The PCR was performed using the following thermal cycling conditions: initial denaturation at 95 ºC for 2 min, denaturation at 95 ºC for 30 sec, annealing 55 ºC for 30 sec, and extension at 72 ºC for 2 min, respectively. Single-pass sequencing was performed in electrophoresis using an ABI 3730xl sequencer. The 16s rRNA sequence with an accession number MK850444.1 was blast using the NCBI similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed by following sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The evolutionary analysis of isolated species was carried out using MEGA 6.0 software.

3.4. Analytical methods

The high-performance liquid chromatography (HPLC) analysis was performed to measure the concentration of phenol and 4-CP. The HPLC (UFLC Shimadzu, Japan) was coupled with

a column (C-18 Shim-pack solar; 4.6 mm× 250 mm) and a Photodiode Array Detector (PDA). A mixture of HPLC-grade methanol and water (75:25 v/v) was used as a mobile phase. The flow rate of the mobile phase was kept at 1.0 mL/min. Before analysis, the samples were filtered through a 0.22 µm cellulose filter.

The chemical oxygen demand (COD), attached biomass, and mixed liquor suspended solids (MLSS) were determined using standard protocol (APHA, 2005). In detail, the COD of the wastewater was evaluated using the dichromate method. The attached and MLSS were analysed using the gravimetric method. Dissolved oxygen (DO) and pH of the wastewater were measured regularly using the DO meter (HD 2109.1; Delta OHM; Italy) and pH meter (HD 2305.0; Delta OHM; Italy), respectively. The bacterial biofilm growth onto the bio carriers was analyzed by SEM (QUANTA 200F, Netherland). The immobilized biocarriers dried at 30 ºC in a vacuum oven overnight. The dried samples were coated with gold for optimum resolution and examined by SEM under low vacuum conditions.

Gas chromatography-mass spectroscopy (GC-MS) analysis of the treated wastewater was carried out to identify the intermediates formed during the biodegradation. Prior to the analysis, the sample was filtered and acidified with HCl (pH up to 2.0-3.0) and extracted with ethyl acetate. Then, the extracted sample was filtered with anhydrous sodium sulphate to remove the remaining moisture. The GC-MS (QP201 Shimadzu, USA) was equipped with RXi-5 Sil MS column (30 m × 0.25 mm × 0.25 µm). Derivatization of the extract sample was performed in a water bath at 60 ℃ for 30 min using N, O-Bis (trimethylsilyl) acetamide (Nie et al., 2016). Helium gas was used as a carrier (flow rate of 1.0 mL/min) and the injector temperature was maintained at 280 ℃. The oven temperature was initially kept at 60 ℃ for 1.0 min followed by linear increment by 15 ℃/min up to 280 ℃ and held for 5 min.

3.5. Biodegradation of phenol in the free cell system

3.5.1. Process variables optimization

The effect of the process parameters on the removal of phenol was studied in Erlenmeyer flasks (250 mL). The process variables, including pH (5.0-9.0), temperature (25-40 ºC), and phenol concentration (50-500 mg/L) were considered for the optimization study. Initially, the concentration of phenol was kept constant at 150 mg/L for the optimization of pH and temperature. During the optimization of one parameter, other parameters were kept constant. Intermediate samples were taken at regular intervals for the determination of phenol concentration.

3.5.2. Growth kinetic models

The substrate inhibition kinetics can be studied by two different mathematical models, such as Monod and Andrew-Haldane. The Monod equation can be expressed as:

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{\mu_{\max} S}{K_s + S} \qquad (2)$$

where μ, x, μ_{max}, S, and K_s represent specific growth rate (h^{-1}), biomass concentration (mg/L), maximum specific growth rate (h^{-1}), phenol concentration (mg/L), and half-saturation constant (mg/L), respectively. Again, the specific growth rate (μ) value can be found out by evaluating the slope of the curve plotted between logarithm value of biomass ratio and time duration as given by Eq. (3) (Hussain et al., 2015):

$$\mu = \frac{\log\left(\frac{x_2}{x_1}\right)}{t_2 - t_1} \qquad (3)$$

where the terms x_1 and x_2 represent the value of cell concentration at time t_1 and t_2, respectively.

The microbial growth under inhibitory conditions can be expressed by Eq. (4) (Geed et al., 2017):

$$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}} \quad (4)$$

where K_i refers to the inhibition constant (mg/L). The kinetic parameters involved in the Haldane model were obtained by a nonlinear least-squares regression method.

3.6. Biodegradation of phenol in a packed bed bioreactor

3.6.1. Packed bed bioreactor set up, immobilization, and operation

In this work, a borosilicate glass reactor was fabricated and used for phenol degradation analysis by *Bacillus* sp. immobilized on packing material. The lab-scale packed bed bioreactor (PBBR) was made of 60 cm in length and 5.5 cm internal diameter with a working capacity of 1000 mL (1425 mL total volume), as given in Table 3.2. The schematic diagram of PBBR is shown in Figure 3.2. Two sampling ports (4 cm and 45 cm from the bottom of the reactor) were functioning as inlet and outlet of the reactor. Air was supplied to the reactor through a stone diffuser by an air compressor (XP-AC-24 L XtraPower). The air was filtered through a 0.22 µm nylon syringe filter to prevent contamination, and its flow rate was controlled by a rotameter (SRS-MG5, Eureka, Pune). The dissolved oxygen (DO) was maintained above 4.0 mg/L in the PBBR throughout the operation.

LDPE has an average density value of 0.930 g/cm^3 and stable below 90 ºC. LDPE sheets were brought from a local shop near Lanka, Varanasi, INDIA, and cut into 1.0 cm^3 cubical shape to use in the reactor as packing material. LDPE cubes were washed with 70% ethanol,

soaked in distilled water, and dried in an oven at 40 ºC overnight. 6.0 g of LDPE cubes were packed in the PBBR along with 1000 mL sterilized MSM with the addition of phenol (100 mg/L) along with bacterial inoculums approximately of 1.2×10^7 CFU/mL (4% v/v). After 15 days, the biofilm developed on the LDPE was confirmed by scanning electron microscopy analysis (SEM). The PBBR was then operated with phenol as the carbon source in a continuous mode by varying the flow rate from 15-60 mL/h at optimized conditions. The efficacy of PBBR was investigated by evaluating removal efficiency (RE), elimination capacity (EC), and inlet loading rate (ILR) as follows (Geed et al., 2017; Yadav et al., 2014):

Elimination capacity (EC) (mg/L/d) = $\frac{C_{in}-C_{out}}{Q}\times V$ (5)

Removal efficiency (%) (RE) = $\frac{C_{in}-C_{out}}{C_{in}}\times 100$ (6)

Inlet loading rate (ILR) (mg/L/d) = $\frac{Q\times C_{in}}{V}$ (7)

where C_{in} (mg/L) and C_{out} (mg/L) correspond to the inlet and outlet phenol concentrations in PBBR, Q (mL/h) refers to the volumetric flow rate of the influent, and V (L) is the working capacity of the reactor.

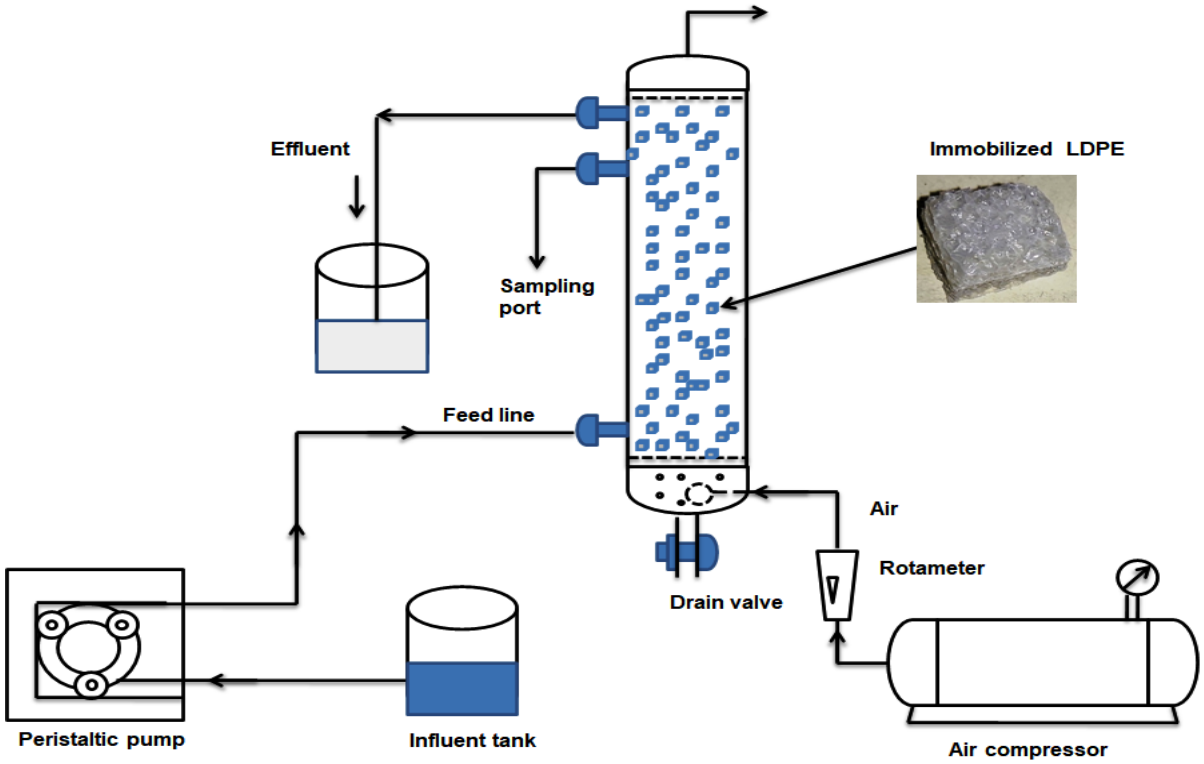

Figure 3.2. Schematic diagram of PBBR set up for the removal of phenol.

Table 3.2. Dimensional specifications, conditions, and outcomes of the PBBR.

Variables	Units	Values
Reactor Material type	-	Borosilicate glass
Outer diameter of the reactor	cm	5.5
Height of the reactor	cm	60.0
Total volume	mL	1425
Packing material type	-	Low density polyethylene
Equivalent diameter of the packing material	cm	1.0
Porosity of the bed	-	0.31
pH of the media	-	7.0 ± 0.2
Temperature	$^{\circ}$C	30 ± 2

Dissolved oxygen	mg/L	4.5 ± 0.5
Initial concentration of phenol	mg/L	100
Flow rate of the solution	mL/h	15-60
Mass of dried immobilized biomass	g	9.5
Estimated value of external surface area per unit weight of dried immobilized cells (a_m)	cm^2/g	4.14

3.6.2. External mass transfer analysis in bioremediation of phenol in a packed bed bioreactor

3.6.2.1. The external liquid film diffusion process

In a static bed bioreactor with immobilized carrier system, substrate transport occurs via two mechanisms, viz. the external mass transfer process, which arises due to the concentration gradient of the substrate between the bulk solution phase to the surface of biocatalyst and the internal mass transport occurs due to the biochemical reaction within the biofilm (Geed et al., 2018; Banerjee and Ghoshal, 2016).

3.6.2.2. Phenol biodegradation rate constant

By considering no back mixing of fluid, cubic shaped packing materials, steady-state, and no axial dispersion in PBBR, the corresponding material balance can be expressed by the following equation (Tepe and Dursun, 2008; Sonwani et al., 2019)

$$\left(\frac{hQ}{W}\right)\frac{dC}{dZ}\times 6\times 10^{-2} = -r \qquad (8)$$

where h represents the height of the reactor (cm), W denotes the total dried biomass immobilized on LDPE (g), Q is the rate of feed inlet (mL/min), and r corresponds to the degradation rate of phenol (mg/g h), respectively. dC/dZ represents the concentration gradient of phenol across the reactor column (mg/L cm). The substrate degsradation rate can be expressed in terms of observed first-order rate coefficient, k_p (L/g h) and substrate concentration, C (mg/L) as following (Sheeja et al., 2002)

$$r = k_p C \qquad (9)$$

Now, combining and integrating the resultant equation obtained from Eq. (8) and Eq. (9), the following equation can be resulted as:

$$k_p = \left(\frac{Q}{W}\right)\times \ln\left(\frac{C_{in}}{C_{out}}\right)\times 6\times 10^{-2} \qquad (10)$$

where C_{in} and C_{out} are the initial and final phenol concentrations (mg/L) along the reactor column, respectively.

3.6.2.3. Combined mass transport kinetics and phenol degradation

The external mass transfer rate may inhibit the degradation rate of phenol in PBBR due to the variation of the external film diffusion rate. Hence, the expression for r_m can be written as;

$$r_m = k_m a_m (C - C_s) \qquad (11)$$

where k_m, a_m, C, C_s, and r_m are substrate diffusion constant (L/cm^2 h), external surface area per unit mass of dried immobilized biomass (cm^2/g), phenol concentration in the solution phase, phenol concentration at the surface of immobilized LDPE (mg/L), and the substrate diffusion rate (mg/g h) respectively.

The kinetic equation for the degradation rate of phenol can be written by Eq. (12):

$$r = k_s\, a_m\, C_s \tag{12}$$

where k_s represent the intrinsic first-order rate constant (L/cm^2 h).

At the equilibrium state, the substrate diffusion coefficient (k_m) can be obtained by comparing and rearranging Eq. (9), Eq. (11), and Eq. (12)

$$k_m = \frac{k_p\, k_s}{(k_s\, a_m - k_p)} \tag{13}$$

3.6.2.4. A model formulation for phenol biodegradation

Generally, k_m can be expressed in terms of process parameters such as column diameter, fluid characteristics (i.e., density, viscosity, diffusivity), and mass velocity by a dimensionless group given as (Banerjee and Ghoshal, 2016)

$$J_D = \frac{k_m\, \rho}{G}\left(\frac{\mu}{\rho\, D_f}\right)^{\frac{2}{3}} = K\, \mathrm{Re}^{n-1} \tag{14}$$

where J_D, Colburn factor is a dimensionless number expressed in terms of Reynolds number. G, ρ, μ, D_f , and n are the average mass velocity (g/cm^2 h), density (g/cm^3), viscosity (g/cm s), and diffusivity (g/cm s) of the wastewater, and exponent respectively. K is a correlation coefficient which depends on mass transfer coefficient and fluid velocity. The superficial flow velocity of the fluid (G) in the PBBR can be calculated by the following equation as

$$G = \frac{Q\rho}{A\varepsilon} \times 60 \quad (15)$$

where A represents the cross-sectional area of the reactor (cm^2).

By simplifying Eq. (14) and rearranging for mass transfer coefficient as (Sonwani et al., 2019)

$$k_m = N\ G^n \quad (16)$$

where N is a parameter which depends on the fluids characteristics. Its value generally varies from 10^{-4}-10^{-1} (Banerjee and Ghoshal, 2016; Sheeja et al., 2002) and can be expressed as:

$$N = \left(\frac{K}{\rho}\right)\left(\frac{\mu}{\rho\ D_f}\right)^{-\frac{2}{3}}\left(\frac{d_p}{\mu}\right)^{n-1} \quad (17)$$

By substituting Eq. (16) into Eq. (13) and rearranging for k_p gives the Eq. (18) as:

$$\frac{1}{k_p} = \left(\frac{1}{\mathrm{N}\ a_m}\right)\left(\frac{1}{G^n}\right) + \left(\frac{1}{k_s\ a_m}\right) \quad (18)$$

To evaluate a_m, the slope ($1/N\ a_m$) and the intercept ($1/k_s\ a_m$) can be obtained from the straight-line graph plotted against $1/k_p$ and $1/G^n$ at different values of n and K.

Then, the estimated a_m values were compared with the experimentally obtained a_m values to predict the best set of K and n values for the adequate external mass transfer correlation phenol degradation in PBBR.

3.7. Phenol biodegradation in a moving bed biofilm reactor

3.7.1. Description of modified carrier and reactor set-up

The MBBR carriers used in this study were composed of polypropylene (PP) and polyurethane foam (PUF). The PP carriers (with length and diameter of 12 mm and 25 mm, respectively) were purchased from the Netrox aqua solution, New Delhi, India. The density and specific surface area of PP were 930 kg/m^3 and 550 m^2/m^3, respectively. The PUF was purchased from a local shop of Varanasi, India and cut into cubic pieces with each side of 2.6 ± 0.2 cm. These pieces were inserted into alternate holes of the PP carriers, and the combined carriers are known as PP-PUF. The average weight of each PP-PUF carrier was 1.187 ± 0.04 g.

The MBBR reactor was made of a borosilicate glass of a total volume 2.0 L (working capacity of 1.8 L). The air diffuser was provided at the bottom of the glass reactor to supply air, which ensures continuous movement of the carriers inside the MBBR. The MBBR was equipped with the air compressor, peristaltic pump, rotameter, and effluent tank, as shown in Figure 3.3. The influent was fed to the reactor by a peristaltic pump. The volume of modified carriers filled in the MBBR was 40 % of the working volume. All the sampling analysis was carried out in triplicate. In detail, the MBBR was fed with MSM along with 10 mL of bacterial inoculum. The bioreactor was operated in batch mode for 20 days to ensure adequate growth of biofilm onto the carriers. After the confirmation of the biofilm layer by Scanning electron microscopy technique, the MBBR was operated at various conditions of pH (5.0 – 8.5), HRT (2.0 – 12.0 h), and AFR (0.8 – 3.5 LPM) with phenol (0.1 g/L), and NH^{4+}-N (0.1 g/L).

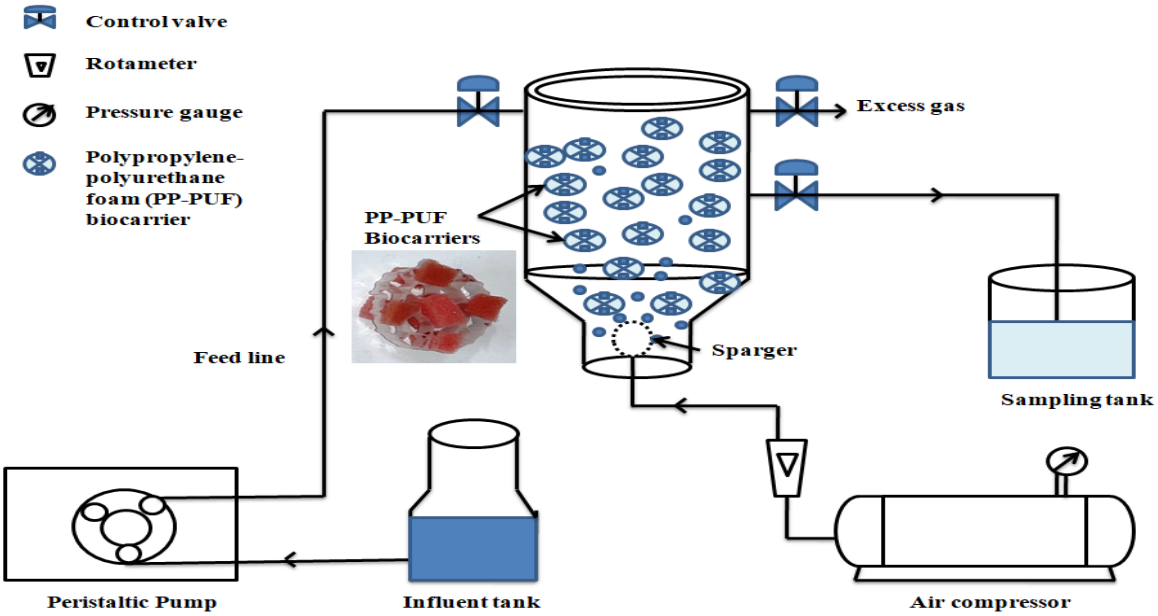

Figure 3.3. A schematic representation of a MBBR for phenol treatment.

3.7.2. Process parameters optimization using response surface methodology

The optimization of experimental parameters was done by central composite design (CCD) of response surface methodology (RSM). RSM is a statistical used to estimate the optimum conditions from a set of designed experiments. The effect of the independent process parameters on the response variables was analyzed using *Design-Expert* software (version 11) (Stat –Ease Inc., Minneapolis, USA). The optimum operating conditions were evaluated by analyzing the obtained values of process parameters through different model tests, including the fit summary analysis, model summary statistics, fit statistics (ANOVA analysis), and model graphs.

3.7.3. Substrate utilization rate kinetics

The kinetic models assist in predicting the performance of bioreactors by determining the substrate utilization rate. It mainly depends on the pH of the wastewater, airflow rate, and types of biocarriers. The MBBR was operated at optimized conditions (pH =6.5, RT=7.0 h, and AFR=2.15 LPM), and intermediate samples were taken to evaluate the transient

concentration of phenol, COD, and ammonia in the wastewater. Thereafter, the kinetic models were applied to analyze the substrate utilization rate in MBBR. These models are also suitable for optimizing and tuning the removal process at the laboratorsy- or pilot-scale reactors (AghaBeiki et al., 2016; Brink et al., 2017). In this study, first-order and second-order (Grau model) models were evaluated to predict phenol, COD, and ammonia removal rate kinetics.

3.7.3.1. First-order kinetic model

The phenol removal, assuming a first-order kinetic, can be expressed as the following equation in a well-agitated system (Brink et al., 2017):

$$\frac{dS}{dt} = -k.\,X.\,S \qquad (19)$$

where S (mg/L) is the final concentration, t is the retention time (h), k is the first-order rate constant (L/mg VSS h), and $X\ (t)$ is the biomass concentration in the MBBR (mg/L). The Eq. (19) cannot be directly integrated to determine $S\ (t)$ as the nature $X\ (t)$ is not known. Therefore, an average biomass concentration ($\bar{X}$) is used in Eq. (19) and is lumped with k to give the final rate law as following

$$\frac{dS}{S} = -\,k.\,\bar{X}.dt = -\,k_1 dt \qquad (20)$$

The degradation rate can be obtained by integrating and linearizing the Eq. (20) as

$$\frac{S_0 - S}{t} = k_1.\,S \qquad (21)$$

where k_1 is the lumped first-order kinetic parameter (h^{-1}). The lumped first-order rate constant (k_1) can be obtained from the slope of the graph plotted between $\frac{S_0 - S}{t}$ against S.

3.7.3.2. Second-order kinetic model (Grau model)

The second-order kinetics for phenol removal can be expressed by Eq. (22) as

$$\frac{dS}{dt} = -k_s . X . S^2 \quad (22)$$

where k_s represents the second-order rate constant (L^2/gVSS. gCOD. h) which depends on the initial pollutant concentration and average biomass density in the MBBR.

As discussed above that the nature of *X (t)* is unknown; an average biomass concentration ($\bar{X}$) is lumped with k_s to give the final rate law as following

$$\frac{dS}{dt} = -(ks . \bar{X})S^2 = -k_2 . S^2 \quad (23)$$

The Eq. (23) can be further integrated to give the following expression as:

$$\frac{S}{S_0} = \frac{1}{1+S_0 k_2 . t} \quad (24)$$

Now, Eq. (25) can be obtained by integrating and linearizing the Eq. (24) as:

$$\frac{S_0 . t}{S_0 - S} = \frac{1}{k_2 . S0} + t \quad (25)$$

where, S_0 is the initial concentration (mg/L), and k_2 represent a lumped second-order rate constant (L/mg VSS. h). $\frac{S_0 - S}{S_0}$ is the phenol removal efficiency and can be indicated as *E*. Again, the term $\frac{1}{k_2 . S0}$ is assumed as a constant denoted as *m*. Hence, Eq. (25) can be represented as

$$\frac{t}{E} = n . t + m \quad (26)$$

where *n* and *m* (h) are the second-order kinetic parameters. The kinetic parameters can be obtained from the slope and intercept of the graph plotted between $\frac{t}{E}$ against *t*.

3.8. Phytotoxicity analysis

The phytotoxicity study is useful to measure the adverse impact of the pollutants on the floras. The phytotoxicity analysis was carried out using *Vigna radiata* seeds and the process flow diagram is shown in Figure 3.4. Prior to the analysis, the seeds were washed with 0.1 % $HgCl_2$ to avoid any fungal infection and further cleaned with double distilled water. The Petri plates were covered with two layers of filter paper and 15 seeds of *Vigna radiata* were kept in each plate. The plates were regularly added with 20 mL of liquid sample (untreated wastewater or treated wastewater). The distilled water was taken as control. Each plate was incubated at 28 ± 2.0 ℃ to evaluate the germination (%) after 2.0 days and phytotoxicity (%) after 7.0 days as per the following expression:

$$\text{Germination (\%)} = \frac{Number\ of\ seeds\ germinated}{No.of\ seeds\ sowed} \times 100 \quad (27)$$

$$\text{Phytotoxicity (\%)} = \frac{Radicle\ length\ of\ control - Radicle\ length\ of\ test\ sample}{Radicle\ length\ of\ control} \times 100 \quad (28)$$

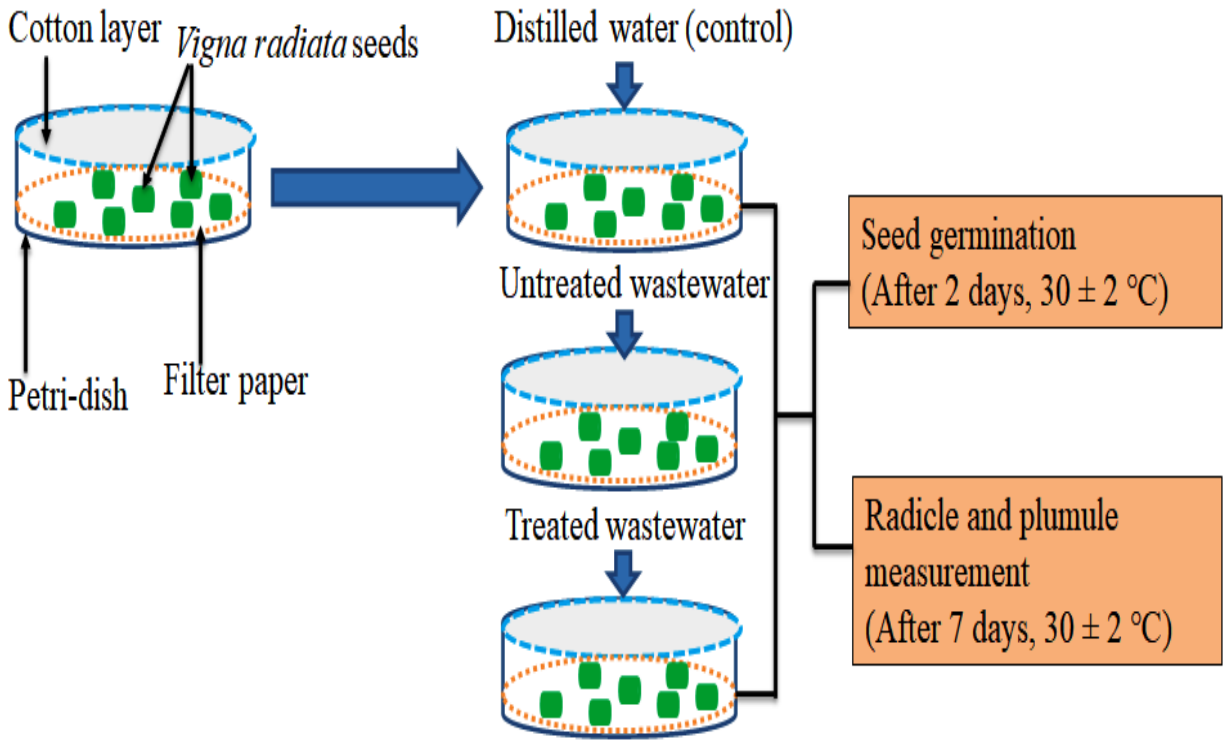

Figure 3.4. Process flow diagram of phytotoxicity analysis in distilled water (control), untreated wastewater, and treated wastewater.

3.9. Effect of biogenic substrate on biodegradation of 4-CP

3.9.1. Bioreactor set-up, immobilization, and operating procedure

A lab-scale MBBR was developed for the biodegradation of 4-CP. The wastewater was fed to the MBBR by a peristaltic pump (Milton Roy, India, A-11 SS). The MBBR was filled with polypropylene – polyurethane foam (PP-PUF) carriers which were developed by inserting polyurethane foam (PUF) into the alternate holes of polypropylene (PP).

The synthetic wastewater containing 4-CP (50 mg/L) as a sole carbon source and bacterial inoculum was fed to the MBBR. The MBBR was operated at fed-batch mode for 20 days to allow the biofilm growth onto the PP-PUF carriers. The wastewater was replaced with a fresh one after complete removal of 4-CP was achieved. The growth of the biofilm was confirmed by the scanning electron microscopy (SEM) technique. After that, the bioreactor was operated at a wide range of 4-CP concentrations, peptone concentrations, and hydraulic retention time (HRT).

3.9.2. Experimental design using response surface methodology (RSM)

The process parameters were optimized by an effective technique, i.e., response surface methodology (RSM). Moreover, it is a cost-effective statistical tool since it saves time and decreases chemicals cost by reducing the number of experiments. In addition, it provides the correlation between the process variables with responses (Sonwani et al., 2020; Faridnasr et al., 2016). The optimization of process parameters such as 4-CP concentration (40 – 100 mg/L), peptone concentration (0 – 0.6 g/L), and hydraulic retention time (HRT) (8.0 – 36 h) was carried out in a MBBR. The Design-Expert software (Version 13) was used for the optimization study.

3.9.3. Kinetic study

Previously, various kinetic models were developed and used to predict the biochemical reaction rate in different bioreactors. In this direction, the Monod and Modified Stover-Kincannon kinetic models are widely used for the biodegradation of toxic compounds in MBBR (Esmaeilirad et al., 2015; Mohammadi et al., 2018). The effluent samples were collected to measure the concentration of 4-CP and COD in the effluent. Finally, the Monod and Modified Stover- Kincannon kinetics were applied to evaluate the substrate utilization rate and microbial growth.

3.9.3.1. Monod model

The Monod model is widely studied to evaluate microbial growth in the bioreactor. For a continuous stirred tank reactor (CSTR), the substrate mass balance can be expressed as follows

$$\frac{dS}{dt} V = Q.F_0 - Q.F + V.r_{su} \quad (29)$$

where V, Q, r_{su} represent the volume of the MBBR (L), feed flow rate of the influent (L/d), and the substrate utilization rate (g/L. d). At steady-state conditions, the change of substrate condition ($\frac{dF}{dt}$) can be neglected. F_0 and F are the initial and final substrate concentration (mg/L), respectively.

Now the Eq. (29) can be rearranged, and the substrate utilization rate can be written as

$$r_{su} = \frac{F_0 - F}{t} = -\frac{k.X.F}{K_S + F} \quad (30)$$

The final expression can be obtained by linearization and inverting Eq. (30) as

$$\frac{t.X}{F_0 - F} = \frac{K_S}{k}\frac{1}{F} + \frac{1}{k} \quad (31)$$

where *t*, K_s, and *k* represent the hydraulic retention time (h), half-saturation constant (mg/L), and maximum specific growth rate (h^{-1}), respectively.

3.9.3.2. Modified-Stover Kincannon model

Earlier in the 1980s, the substrate degradation kinetic in the biofilm reactors was predicted by Stover and Kincannon. The original Stover-Kincannon model was used to estimate the substrate utilization rate for rotating biological contactors (RBCs), which can be expressed as

$$\frac{dF}{dt} = \frac{U_{max}\left(\frac{Q.\,F_0}{A}\right)}{K_B + \left(\frac{Q.\,F_0}{A}\right)} \tag{32}$$

where U_{max}, K_B, A represent the maximum substrate utilization rate (mg COD/ L. d), saturation constant (mg COD/ L. d), and surface area of the disc support. This kinetic model was later improved by replacing the surface area of the active biomass with the working volume of the MBBR due to the limitation of active biomass area measurement in the bioreactor (Mohammadi et al., 2018; Brink et al., 2017). Hence, the modified expression for the substrate utilization rate can be written as

$$\frac{dF}{dt} = \frac{Q}{V}(F_0 - F) = \frac{U_{max}\left(\frac{Q.\,F_0}{V}\right)}{K_B + \left(\frac{Q.\,F_0}{V}\right)} \tag{33}$$

Now, by linearizing Eq. (33) gives the final expression as follows:

$$\frac{V}{(F_0 - F).Q} = \frac{K_B}{U_{max}}\left(\frac{V}{Q.\,F_0}\right) + \frac{1}{U_{max}} \tag{34}$$

Based on Eq. (34), the graph plotted between $\frac{V}{(F_0 - F).\,Q}$ against $\frac{V}{Q.\,F_0}$ gives the values of kinetic parameters.

3.10. Comparative analysis of a packed bed bioreactor and a moving bed bioreactor for 4-chlorophenol Biodegradation

3.10.1. Experimental set-up: bioreactor start-up and operation

MBBR and PBBR were fabricated of cylindrical borosilicate glass (Figure 3.5). The details of the bioreactors are provided in Table 3.3. The working volumes of PBBR and MBBR were 1.0 L each. Each bioreactor was filled up with 40 % volume of PP-PUF biocarriers. The bioreactors were equipped with an air compressor, peristaltic pump, rotameter, feed tank, and effluent tank. The stone diffusers were provided at the bottom of the bioreactors for air distribution. The bioreactors were operated at room temperature 30 ± 2.0 °C.

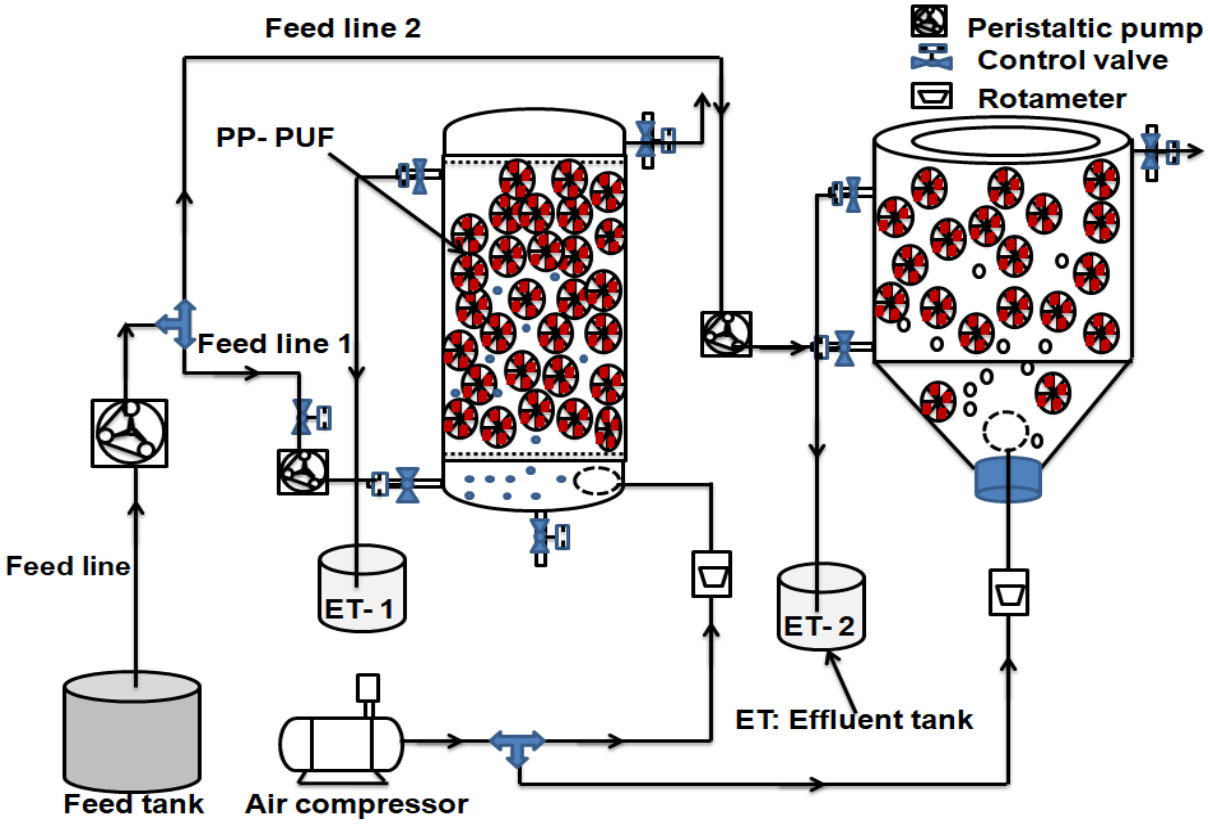

Figure 3.5. Schematic representation of moving bed bioreactor (MBBR) and packed bed bioreactor (PBBR) for 4-CP biodegradation.

Table 3.3. The design details of MBBR and PBBR and characteristics of the biocarrier.

	Bioreactor design configuration	
Features	MBBR	PBBR

Bioreactor material type	Borosilicate glass	Borosilicate glass
Total volume (L)	1.6	1.4
Working volume (L)	1.0	1.0
Height of the bioreactor (cm)	20	60
Biocarrier filling ratio (%)	40	40
	Biocarrier characteristics	
Material	PP-PUF	PP-PUF
Density (kg/m^3)	940	940
Length (mm)	25	25
Diameter (mm)	12	12
Mass of 1 m^3 (kg)	75	75
Specific surface area (m^2/m^3)	550	550

PP-PUF: Polypropylene-polyurethane foam

The bioreactors were fed with synthetic wastewater containing 50 mg/L of 4-CP followed by inoculation with isolated bacterial culture (3 % v/v). The bioreactors were initially operated at fed-batch mode for 8 weeks. The DO was maintained at 4 ± 1.5 mg/L by adjusting the airflow rate. The biofilm growth onto carriers was confirmed by scanning electron microscopy (SEM) analysis. After that, the bioreactors were switched to continuous mode. The whole operation was divided into two stages. In the 1st stage, the hydraulic retention time (HRT) was decreased from 48 to 10 h at an initial 4-CP concentration of 50 mg/L. In the 2nd stage, the inlet loading rate (ILR) was increased by increasing the initial concentration of 4-CP from 50 mg/L to 250 mg/L. The summaries of operating conditions are reported in Table 3.4. The operating conditions were changed as the steady-state performance was achieved. The samples were taken at regular time intervals for the determination of COD, concentration of 4-CP, and biomass growth (attached and suspended).

Table 3.4. The summary of operational conditions in different runs in continuous bioreactors

Stage	Run of operation	4-CP concentration (mg/L)	HRT (h)	ILR (kg/m^3. d)
	1	50	48	0.025
	2	50	36	0.033
Stage 1	3	50	24	0.05
	4	50	16	0.075
	5	50	10	0.12
	6	50	36	0.033
	7	100	36	0.067
Stage 2	8	150	36	0.1
	9	200	36	0.133
	10	250	36	0.167

HRT: Hydraulic retention time; ILR: Inlet loading rate