2.6. Objective of the research work

The overall objective of the present work is to develop an efficient, cost-effective, environmentally sound technique for effective treatment of dyes and PAHs. Therefore, the objectives of this work follows as:

1. Acclimatization, isolation, and screening of potential bacterial species having Congo red dye and naphthalene degrading potential.
2. Identification of potential bacterial species using 16S rRNA technique and its application in the biodegradation of Congo red dye and naphthalene.
3. Optimization of process parameters using one variable a time (OVAT) and RSM techniques.
4. Performance evaluation of bioreactors, namely Integrated Aerobic Treatment Plant (IATP), Packed Bed Bioreactor (PBBR), and Moving Bed Biofilm Reactor (MBBR).
5. Evaluation of the kinetics of Congo red dye and naphthalene degradation using various models.

CHAPTER 3

Process optimization of naphthalene biodegradation using surface response methodology: Kinetic study and performance evaluation of an integrated aerobic treatment plant

CHAPTER 3

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3.1. Introduction

Naphthalene is a type of polycyclic aromatic hydrocarbon (PAH). It is considered a persistent organic pollutant due to its adverse impact on human health and ecological systems (Sakulthaew et al., 2015). The details of PAHs and their adverse impact on human health and ecological systems have been well described in chapters 1 and 2. It is the global prerequisite to explore an effective, environmentally benign, and economical technique to remediate the PAHs. Naphthalene biodegradation by microorganisms such as *Bacillus fusiformis*, *Nocardia otitidiscaviarum*, *Micrococcus* sp., *Streptomyces* sp. etc., have been studied by previous researchers (Jegan et al., 2010; Lin et al., 2014; Seoud et al., 2003). Although there are lots of research works going on in the field of biodegradation but slow degradation rate is a major challenge. The adaptation and enrichment of microorganisms from specific organic sources were significantly improved the biodegradation rate (Yeom et al., 1997). Further, the application of response surface methodology (RSM) in PAHs biodegradation is an underexplored area and can enhance a greater yield with fewer experiments than the conventional method. Also, very limited studies are available on naphthalene biodegradation in the continuous bioreactor.

The objective of this study was to isolate the potential bacterial species from petroleum-contaminated soil for naphthalene biodegradation. Naphthalene was taken as a model pollutant. The effects of process variables like pH, initial naphthalene concentration, and temperature were optimized using the central composite design (CCD) of RSM to achieve the optimum operating conditions. A laboratory-scale Integrated Aerobic Treatment Plant (IATP) was operated in continuous mode to study the effect of inlet loading rates (ILRs). In addition, the biodegradation kinetics were analyzed by Monod and Teissier-Edwards models. The phytotoxicity test was performed using *Cicer arietinum* seeds to study the effect of treated wastewater on plant growth.

3.2. Materials and methods

3.2.1. Chemicals and materials

An analytical grade Naphthalene (CAS number 91-20-3) was procured from Sigma-Aldrich, India. All other high purity and analytical grade chemicals used in the present study were purchased from Merck, HiMedia laboratories Pvt. Ltd., SRL chemicals, Sigma Aldrich, India. A non-ionic surfactant (i.e., Triton X-100) was used to enhance the solubility of aromatic hydrocarbons (Lamichhane et al., 2017). The culture media or mineral salt media (MSM) used in the naphthalene biodegradation study was made of the following composition in distilled water (g/L); $K_2HPO_4.2H_2O$ (1.0), KH_2PO_4 (1.0), NaCl (0.5), $MgSO_4.7H_2O$ (0.3), and $(NH_4)_2SO_4$ (0.3). It also contained trace elements of following compositions (g/L); $CaCl_2$ (0.02), $FeCl_3$ (0.002), $MnSO_4.H_2O$ (0.005), $ZnSO_4$ (0.005), $(NH_4)_6Mo_7O_{24}$ (0.001), and 0.2% (w/v) of glucose. The simulated wastewater used in the biodegradation study was prepared by adding MSM and the required amount of naphthalene in distilled water. The final pH of wastewater was adjusted to be 7.0±0.2 by adding sulfuric acid (0.1N-H_2SO_4) and sodium hydroxide (0.1N- NaOH). The wastewater was sterilized at 121 °C for 20 minutes, prior to its biodegradation study.

The sterilized polybags, scissors, parafilm, filter papers (Whatman), syringe, micro tip, micro-tip box, micro-pipette, Eppendorf centrifuge tube, gloves, trays, funnels silicon tube, inoculum loops, cotton rolls, etc. were purchased from Gyan scientific Varanasi, India. The glassware was made of borosilicate glass. All glassware was dried in an oven (NSW, India) at 105 °C for 30 minutes before its application.

3.2.2. Collection of soil sample for the isolation of bacterial species

It is well known that the potential aromatic hydrocarbons degrading bacterial species should be present or survive in petroleum-contaminated soil and wastewater samples (Shen et al., 2015; Kureel et al., 2017). For example, Shen et al. (2015) have isolated four bacterial species (i.e., *Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp., and *Pseudomonas* sp.) from the Shengli oilfield polluted sludge and reported that these species were capable of degrading the PAHs (i.e., naphthalene, phenanthrene, pyrene). Therefore, the petroleum-contaminated soil samples and activated sludge were obtained from the near site of Indian Oil Corporation Limited (IOCL) refinery, Mathura (Uttar Pradesh), India (27°30′12″ N, 77°40′19″ E and 181 m elevation above sea level), for the isolation of bacterial species. This refinery is continuously working on the production of various aromatic hydrocarbons based products and released effluents which contaminate the soil and water of near site. The samples were collected in sterile polyethylene bags and stored at 4 °C for further application.

3.2.3. Enrichment and isolation of potential bacterial species

Initially, petroleum-contaminated soil (10 gm) was enriched with MSM (100 mL) and naphthalene (10 mg/L) in an Erlenmeyer flask (250 mL). This flask was kept in the rotary incubator (NSW 256, India) at 150 rpm and 35±2 °C for seven days. After that, 10 mL of sample was taken from the incubated flask and further added into another flask, which contained fresh MSM (100 mL) and increased amount of naphthalene and followed by incubation under similar conditions. This process was repeated thrice with gradually increasing the naphthalene concentration. After enrichment, the serial dilution followed by the pour-plate method was used to isolate the naphthalene degrading bacterial species. A process flow diagram of enrichment and isolation of bacterial species is shown in **Figure 3.1**. The screening of potential bacterial species was performed by growing on nutrient-agar plates at 35±2 °C for 48 h. Finally, ten pure bacterial species, namely RKS1, RKS2, RKS3, RKS4, RKS5, RKS6, RKS7, RKS8, RKS9, and RKS10 were isolated and stored at 4 °C for further application in the biodegradation study.

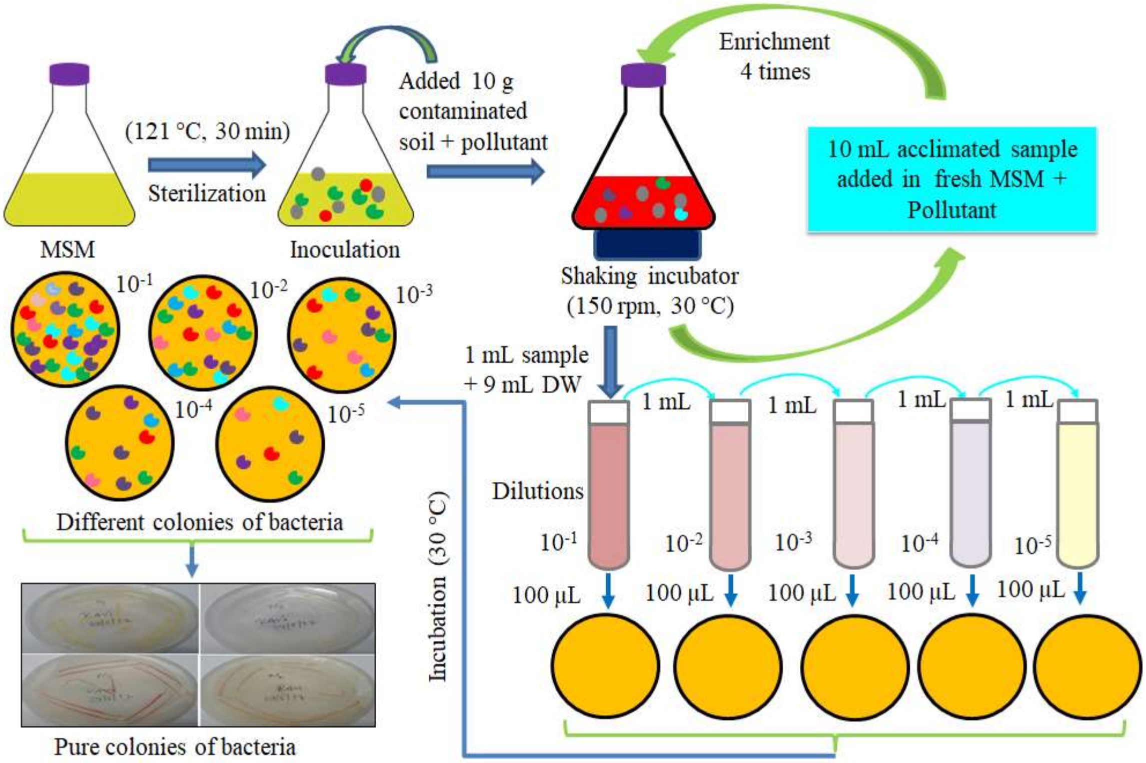

Figure 3.1. Process flow diagram of enrichment and isolation of bacterial species.

3.2.4. Identification of bacterial species and phylogenetic tree construction

The characterization of isolated bacterial species was performed at Triyat Scientific, Nagpur, India. The purification of DNA was carried out using the basic protocol (Geed et al., 2017). The amplification of 16S rRNA gene was performed using the forward (27F-5′ AGAGTTTGATCMTGGCTCAG3′) and reverse (1492R-5′ TACGGYTACCTTGTTACGACTT3′) primers. Polymerase Chain Reaction (PCR) mixture (25 µL) was made of the following composition: 5.0 µL of isolated DNA, 1.5 µL of forward and reverse primers, 5.0 µL of deionize water, and 12 µL Taq master mixer (Taq DNA polymerase, 0.4 mM dNTPs, 3.2 mM $MgCl_2$, and 0.02% bromophenol blue).

PCR is a process in which primers are used to amplify specific genomic DNA sequences with the use of a unique enzyme. This was carried out in a Thermocycler (Bio-rad, India). The PCR was performed in three stages; (a) Initial denaturation at 94 °C for 30 seconds, (b) annealing at 60 °C for 30 seconds, and (c) finally extension at 72 °C for 60 seconds. The obtained PCR products were sequenced using ABI 3730xl sequencer (Applied Biosystems, USA). The sequences were submitted in the GenBank database with the help of BLAST program of NCBI (National Centre for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/blast). The sequences were aligned with MUSCLE alignment tool, and the evolutionary study was performed using the neighbour-joining (NJ) methods. The phylogenetic tree was constructed using MEGA 7.0 software.

3.2.5. Selection of potential bacterial species

For the selection of potential bacterial species, the set of batch experiments were performed in 250 mL Erlenmeyer flasks with free cell culture of bacterial species. Initially, the pure bacterial species (i.e., RKS1, RKS2, RKS3, RKS4, RKS5, RKS6, RKS7, RKS8, RKS9, and RKS10) were grown in nutrient broth. The flasks contained MSM (100 mL) and naphthalene (10 mg/L) were inoculated with 1.0 mL of respective bacterial inoculum. An abiotic sample was also used as a control. All flasks were incubated at 35±2 °C in the rotary incubator at 150 rpm for 5 days. All the experiments were carried out in triplicates. The samples were taken at the regular interval for further analysis, and most potential bacterial sp. was used for the naphthalene degradation.

3.2.6. Design of experiments using response surface methodology

RSM is a suitable method to study the interactive relationship between factors and response variables. It is also widely used to optimize the process variables, including pH, temperature, carrier filling ratio, pollutant concentration, etc. with minimum efforts (Sonwani et al., 2019b). Gusain et al. (2016) have reported that the RSM is an effective technique to obtain the high yield with minimum effort than one variable at a time (OVAT) technique. In the present study, a Design Expert 11 software (Stat-Ease Inc., Minneapolis, USA) was used to optimize the process variables. The total number of experiments were performed with the following terms: $2^n + 2n + n_o$. Where n and n_o represent the number of independent variables and repetitions of experiments at the center point, respectively. A polynomial equation was used to analyze the experimental data (Eq. 3.1).

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_{ij} + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \ldots \tag{3.1}$$

where Y is the response (naphthalene removal efficiency %), β is the correlation coefficient, and i and j is the multi-degree coefficients. The experimental data were analyzed with ANOVA (analysis of variance) to obtain the adequate model (i.e., linear, interaction, quadratic, and cubic). Based on the ANOVA analysis, the most suitable model was selected via values of coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}). Furthermore, the model validity was estimated by F-test with probability levels of $p \leq 0.05$.

The set of batch experiments were carried out in 250 mL Erlenmeyer flask to optimize the process parameters. The most affecting process variables, namely pH (5.0-9.0), temperature (25-40 °C), and naphthalene concentration (10-50 mg/L) were optimized using CCD of RSM. For this, twenty sets of experiments were designed by CCD of RSM and summarized in **Table 3.1**.

Table 3.1 Experimental ranges of independent variables used in optimization process.

Factor	Name	Units	Minimum	Maximum	Mean
A	Concentration	mg/L	10.0	50.0	30.0
B	pH	-	5.0	9.0	7.0
C	Temperature	°C	25.0	40.0	32.5

3.2.7. Biodegradation study in integrated aerobic treatment plant

A lab-scale pilot plant (two-stage IATP (TAE/1000 Pignat, France)) was run in continuous mode to scale up the batch process (**Figure 3.2**). The first stage of the bioreactor (R1) was made of polyvinyl chloride (PVC) sheet having the rectangular tank (60.0 L) along with removal lid which had provision of insertion of the measurement probes (DO, pH, and temperature). The bioreactor (R1) was aerated by an air pump (KNF, Labport, Germany) and stirred by a Rushton turbine at 150 rpm. The second stage of the reactor (R2) of integrated system was static settler (30 L) with a removal lid to allow the measurement probes to be positioned in their holders. The wastewater was kept in a storage tank having the capacity of 200 L. The peristaltic pump (Watson Marlow 323E/D) was used to transfer the wastewater in IATP. The pilot plant was operated under optimum operating conditions.

The synthetic wastewater was supplied from a storage tank to the first stage of the reactor (R1) with increasing the flow rate from 200 mL/h to 1000 mL/h. The treated effluent from R1 was sent to the reactor (R2) in series for further biodegradation. The R2 was also used as a settler for the sludge removal.

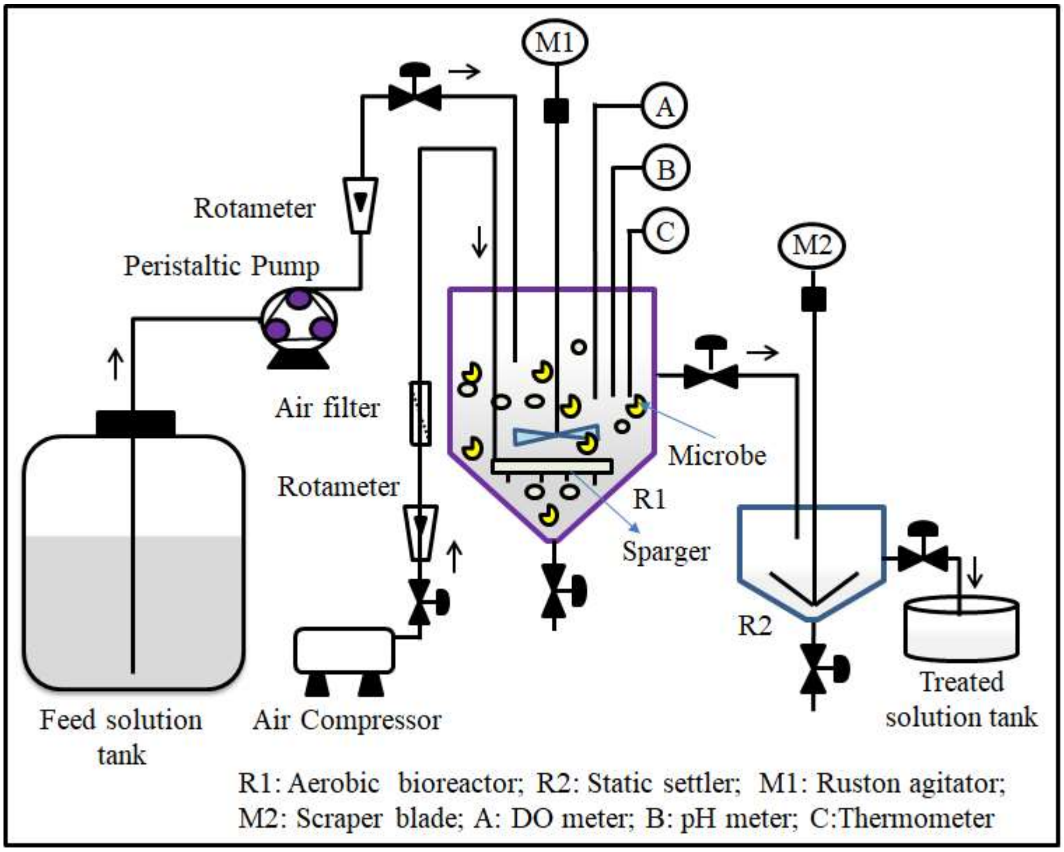

Figure 3.2. Schematic diagram of the pilot-scale integrated aerobic treatment plant.

The treated wastewater samples were taken at regular intervals for the analysis of residual naphthalene. IATP was operated continuously, and variables such as DO, temperature, and pH were regularly monitored. The performance of IATP was calculated using the following equations (Kureel et al., 2017).

$$\%\ \text{Removal efficiency (RE)} = \frac{C_{in} - C_{out}}{C_{in}} \times 100 \qquad (3.2)$$

$$\text{Elimination capacity (EC)} = \frac{(C_{in} - C_{out}) \times Q}{V} \qquad (3.3)$$

$$\text{Inlet loading rate (ILR)} = \frac{C_{in} \times Q}{V} \qquad (3.4)$$

where C_{in} and C_{out} represent the inlet and outlet concentrations of naphthalene (mg/L), Q is the inlet volumetric flow rate (mL/h), and V represents working volume of the reactor (L).

3.2.8. Analytical methods

The residual concentration of naphthalene was measured by a high-performance liquid chromatography (HPLC) (ELICO HL 460, India) (Agrawal and Shahi, 2017). It was equipped with a UV-Vis detector (HD 469) and Xtimate C-18 column (4.6mm×250mm×5.0 μm). The HPLC grade acetonitrile and water with a proportion of 80:20 were mixed under ultra-sonication (25° C for five minutes) and used as a mobile phase with the flow rate of 1 mL/min. Before analysis, all samples were subjected to centrifugation (6000×g, 15 min) and filtered through 0.22 μm of filtration unit (GSWP, Merck Millipore Ltd). A 20 μL sample was manually injected into the injection port, and UV-Vis detector was kept at the wavelength of 254 nm (Zeinali et al., 2008).

GC–MS has been most widely used for the identification of intermediate metabolites formed during biodegradation of the pollutants (Umar et al., 2017). The analysis of intermediates was carried out at AIRF-JNU, New Delhi, using GC–MS (QP201 Shimadzu, USA) equipped with Rxi-5 Sil MS column (30 m X 0.25 mm. X 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature was kept at 260 °C with an initial oven temperature at 50 °C for 3.0 min and temperature was increased up to 150 °C with 5.0 °C/min, and finally increased up to 280 °C with 10 °C/min rate. The observed MS peaks were identified from the existing NIST (National Institute of Standards and Technology) database. The samples were separated using liquid-liquid extraction (LLE) technique (Pugazhendi et al., 2017). The supernatant was twice extracted in an equal volume of ethyl acetate and pass through anhydrous sodium sulphate to remove the remaining moisture.

The concentration of bacterial cell growth was measured by UV-Vis spectrophotometer (SL-2010 ELICO, India). The samples were centrifuged at 5000×g for 10 min. The pellets were used to estimate the bacterial bio-mass. The absorbance of bacterial cell growth was measured at the wavelength of 600 nm (Hazrati et al., 2015). The DO meter (HD 2109.1; Delta OHM; Italy) and pH meter (HD 2305.0; Delta OHM; Italy) were used to monitor dissolved oxygen and pH, respectively. A thermometer was used to measure the temperature.

3.2.9. Kinetic growth model

The kinetic growth models play a key role in the design and modeling of bioreactors. Previously, various kinetic growth models have been used to estimate the specific growth rate of microorganisms for the biodegradation of various organic pollutants (Kureel et al., 2018; Sonwani et al., 2020b). These kinetic growth models can broadly be classified as structured and unstructured models (Muloiwa et al., 2020). The structured models are based on genetic and morphological information and more appropriate for unsteady-state (Dong et al., 2015). On the other hand, the unstructured models designate the specific growth rate of the microorganism (based on microbial biomass and substrate concentration) and are useful under steady-state (Muloiwa et al., 2020). In this chapter, unstructured kinetic growth models are used. The biomass growth rate of microorganisms in the bioreactor is generally estimated by the following expression (Eq. 3.5).

$$\frac{dX}{dt} = r_g = \mu X \qquad (3.5)$$

where r_g is the growth rate of biomass (g/L·d), X is the biomass concentration (mg/L), μ is the specific growth rate (per day), and t is the time (day).

Monod growth model is a well-known expression for estimating the microbial growth rate, especially when describing the single substrate biodegradation (Knightes and Peters, 2006). Two forms of Monod model is denoted by the following equations (Muloiwa et al., 2020):

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{3.6}$$

where μ_{max} is the maximum specific growth rate of microorganisms (per day) and K_s represents the half-saturation rate constant (mg/L), and S is the substrate concentration (mg/L).

Monod model is applicable under the following assumption; (1) single growth-limiting substrate, (2) non-substrate inhibition, (3) does not account for lag phase, (4) at low substrate concentration (Muloiwa et al., 2020). The high substrate concentration in bioreactor could lead to substrate inhibition in microorganisms, and under this condition, Monod model fails to estimate the specific growth rate. Thus, the modified form of Monod models, namely Teissier-Edwards (Eq. 3.7) is widely preferred to address the substrate inhibition under high substrate concentration. Teissier-Edwards model accounts for the substrate inhibition under high substrate concentration.

$$\mu = \mu_{max}\left(e^{-\frac{S}{K_i}} - e^{-\frac{S}{K_s}}\right) \tag{3.7}$$

where K_i is the substrate inhibition constant (mg/L).

3.2.10. Phytotoxicity study

The wastewater containing pollutants reveals adverse effects on humans in terms of toxicity to agriculture crops (Bankole et al., 2018). Therefore, it is necessary to study the toxicity effect of untreated and treated wastewater on crop plant. The germination of plant seed is considered as a rapid technique to study the toxicity effect of wastewater (Kumar et al., 2019). In this study, the phytotoxicity test was performed using *Cicer arietinum* (Chana) seeds. The Petri plates (14 mm × 90 mm), Whatman filter papers (90 mm in diameter), and cotton were taken for the seed germination test (**Figure 3.3**).

Initially, 15 seeds of *Cicer arietinum* were kept between two filter paper and soaked in 15 mL of distilled water (control), untreated and treated wastewater in three separate Petri plates (Kumar et al., 2019). The filter papers were covered with the cotton layer. These plates were kept at 30±2.0 °C for 15 days. The seed germination was recorded after 2 days, and the length of root (cm) and shoot (cm) were estimated after 15 days. The percent of germination was calculated by the following equation:

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sowed}} \times 100 \quad (3.8)$$

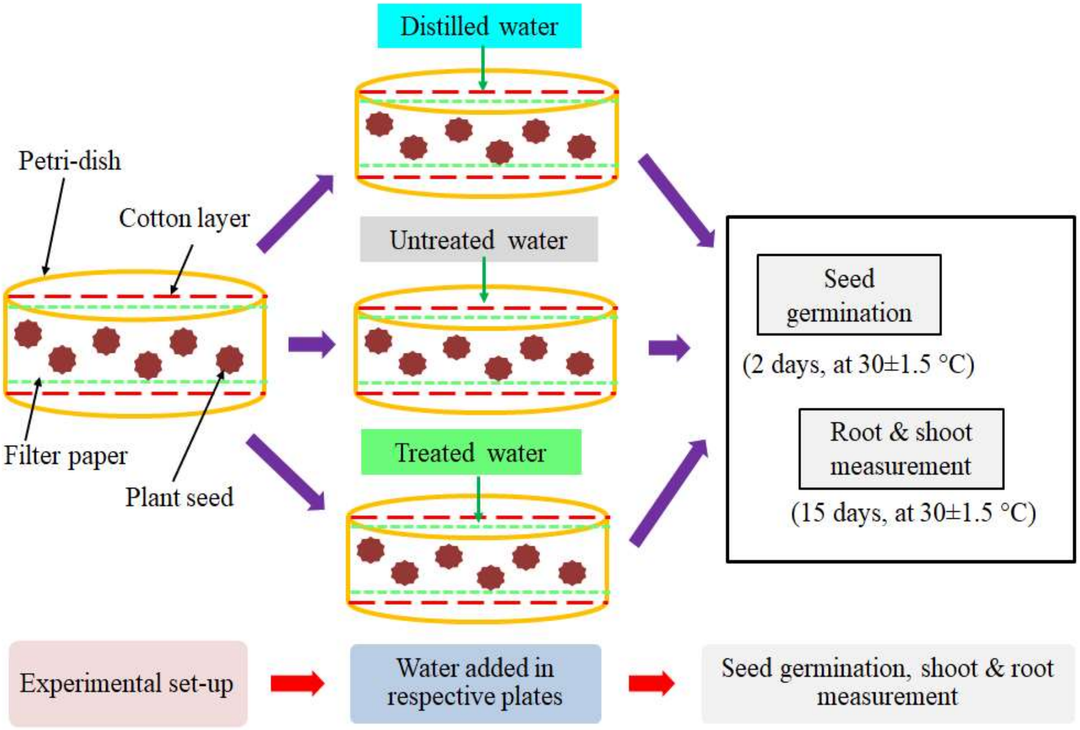

Figure 3.3. Process flow diagram of phytotoxicity analysis in distilled, untreated, and treated wastewater.

3.3. Results and discussion

3.3.1. Selection of potential bacterial species

The removal efficiency (RE %) of naphthalene with respect to the pure culture of bacterial species (RKS1, RKS2, RKS3, RKS4, RKS5, RKS6, RKS7, RKS8, RKS9, and RKS10) is shown in **Figure 3.4.** It can be seen from the figure that the bacterial species

RKS3, RKS4, and RKS8 were found to be more effective for naphthalene removal as compared to the other bacterial species. The REs of 87.8%, 95%, and 85.8% were obtained by species RKS3, RKS4, and RKS8, respectively. Moreover, species RKS4 reveals the highest RE, and hence RKS4 was selected as the most potential bacterial species in the present study.

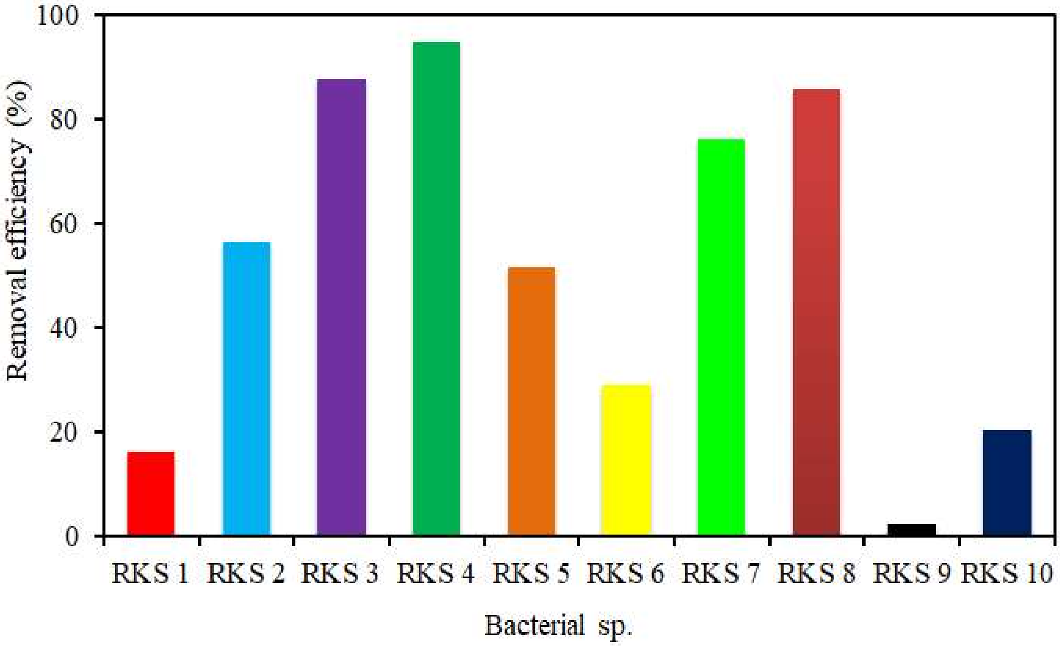

Figure 3.4. Removal efficiency of naphthalene by various bacterial species.

3.3.2. Characterization of potential bacterial species

Bacterial species (RKS4) having maximum naphthalene degradation potential was characterized by 16S rRNA technique. Based on the 16S rRNA molecular identification, the strain had shown the maximum similarity with *Bacillus cereus*, and the sequence was submitted in Gen bank database with an accession number of MH681588.1. **Figure 3.5** represents the phylogenetic tree of isolated bacterial species (i.e., *Bacillus cereus* RKS4 (MH681588.1)) constructed by the maximum likelihood method using MEGA7 software (Tamura et al., 2011). The number of *Bacillus* strains such as *Bacillus cereus*, *Bacillus fusiformis*, *Bacillus species* has been previously reported for the degradation of PAHs in wastewater (Lin et al., 2010; Samanta et al., 2002; Shukla et al., 2014). The literature is in good agreement with our present finding that isolated *Bacillus cereus* showed the potential for naphthalene (PAH) degradation.

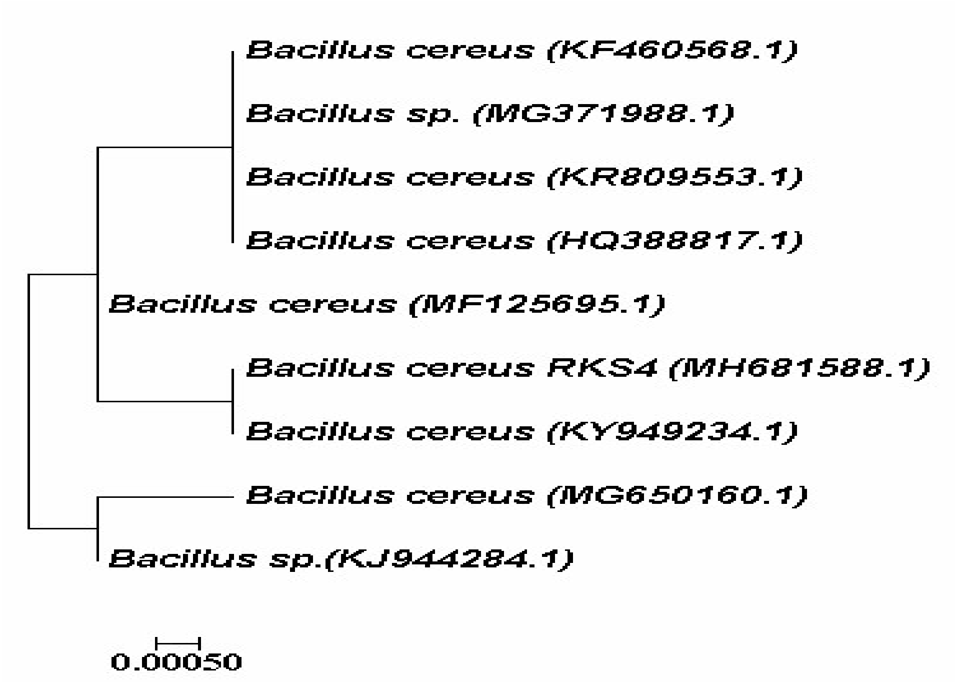

Figure 3.5. Phylogenetic tree of the isolated *Bacillus cereus* RKS4 (MH681588.1).

3.3.3. Analysis by response surface methodology (RSM)

The biodegradation of naphthalene based on the design matrix of CCD of RSM was performed for acquiring the response. The obtained results of the response have been shown in **Table 3.2**. The correlation between the response (naphthalene removal %) and independent variables has been given in the form of a second-order polynomial (Eq. 10).

$$\% \text{ Naphthalene removal} = 85.46 - 7.99A - 4.06B - 9.62C - 2.96AB + 5.79AC + 2.06BC + 2.58A^2 - 24.46\mathrm{B}^2 - 24.47C^2 \quad (3.9)$$

The values of regression coefficient (R^2), adjusted R^2, and predicted R^2 were obtained as 0.988, 0.978, and 0.928, respectively. The obtained values of R^2 signify that the quadratic model was valid for the biodegradation of naphthalene by *Bacillus cereus* RKS4. A similar kind of optimization work has been accomplished by Gusain et al. (2016). They reported that the value of regression coefficient (R^2) more than 0.8 denotes the regression model was well fitted with experimental data.

ANOVA was used to study the significance of the process variable and find out the consistency of the model. The significance of each term was evaluated by the value "*f*" and "*p*". The smaller value of p (<0.05) and the larger value of f denote that the coefficient is more significant (Gusain et al., 2016; Silva et al., 2018). More significant coefficients are the ones that have the probability value (p) less than 0.05. Thus linear effects, namely naphthalene concentration, pH, and temperature were more significant as their p-values were less than 0.05 (**Table 3.3**). The square effects of pH and temperature were also significant. The sign of subtraction or addition in the Eq. (3.9) represents the nature of the variable. Naphthalene concentration has a negative sign prior to its coefficient, which represents that RE was enhanced with decreasing the naphthalene concentration. Temperature and pH have also subtraction sign prior to its coefficient. The magnitude of the coefficient indicates the significance of a particular variable. In the present study, the temperature has the largest value (-9.62) of coefficient among other variables. So, the temperature was the most prominent variable followed by pH and initial naphthalene concentration.

Table 3.2 Experimental design for naphthalene biodegradation by *Bacillus cereus* RKS4 (MH681588.1) based on CCD of RSM.

Run	Conc. (mg/L)	pH	Temp. (°C)	Removal efficiency (%)
1	30	7	32.5	85.31
2	50	5	25	44.66
3	50	7	32.5	82.62
4	10	5	25	66.41
5	10	9	40	31.8

6	50	9	25	27.02
7	30	7	32.5	86.95
8	10	7	32.5	95.87
9	30	7	32.5	87.06
10	30	7	32.5	82.86
11	30	9	32.5	56.16
12	10	9	25	61.78
13	30	7	25	66.45
14	10	5	40	29.36
15	50	9	40	21.37
16	50	5	40	29.62
17	30	7	40	57.95
18	30	7	32.5	81.9
19	30	7	32.5	83.85
20	30	5	32.5	68.65

Table 3.3 Analysis of variance for quadratic model for naphthalene removal (%) by *Bacillus cereus* RKS4 (MH681588.1).

Source	Sum of squares	Mean square	*F*-value	*p*-value
Model	10881.06	1209.01	96.12	< 0.0001
A-Concentration	638.88	638.88	50.79	< 0.0001
B-pH	164.59	164.59	13.09	0.0047
C-Temperature	925.83	925.83	73.60	< 0.0001
AB	70.21	70.21	5.58	0.0398
AC	268.42	268.42	21.34	0.0010
BC	33.87	33.87	2.69	0.1319
A^2	18.25	18.25	1.45	0.2561
B^2	1618.99	1618.99	128.71	< 0.0001
C^2	1646.46	1646.46	130.89	< 0.0001
Residual	125.79	12.58		
Lack of Fit	102.85	20.57	4.48	0.0626
Total	11006.85			

3.3.4. Interactive effect of process parameters

3.3.4.1. Effect of pH and naphthalene concentration

The interactive effect of process variables has been represented by three-dimensional surface and contour plots (**Figure 3.6**). These plots provide desirable information regarding experimental design. The surface and contour plots in **Figure 3.6a** and **3.6b** demonstrate the interactive effect of pH and initial naphthalene concentration on the RE of naphthalene. As the pH of the solution was varied either acidic or alkaline and simultaneously enhanced the initial naphthalene concentration, the RE was sharply declined. At pH 7.0, the RE of 95.8% was obtained at 10 mg/L of naphthalene, while RE was decreased to 82.8% at 50 mg/L. The decrease in the RE at high concentration naphthalene may be due to substrate inhibition and toxicity of naphthalene to microorganisms (Lu et al. 2011; Sonwani et al., 2019a).

Kureel et al. (2017) have reported that the RE of aromatic hydrocarbon (benzene) was decreased due to substrate inhibition at the high concentration of benzene (400 mg/L). At fixed initial naphthalene concentration (30 mg/L), the RE of 86.9% was obtained at pH 7.0. However, at similar conditions, the RE was decreased to 68.6 and 56.1 % at pH 5.0 and 9.0, respectively. The microorganisms produce enzymes with ionic groups on their active cell sites, which should be in an appropriate state (acidic or basic) to function. The variation of the solution pH results in changes in the ionic form of the active cell site, affecting the activity of the enzyme as well as the reaction rate (Ghosal et al., 2016). Lin et al. (2010) have studied the biodegradation of naphthalene at various pH using *Bacillus fusiformis* and reported that the naphthalene RE was decreased at highly acidic as well as at alkaline conditions.

3.3.4.2. Effect of temperature and naphthalene concentration

The surface and contour plot of RE of naphthalene (%) vs. temperature and initial naphthalene concentration is shown in **Figure 3.6c** and **3.6d**. The plots showed that the maximum naphthalene RE was obtained at low concentration (10 mg/L) and moderate temperature (32.5 °C). The RE of naphthalene was increased from 66.4 to 86.9% with rise in temperature from 25 to 32.5 °C. Further, as we increased temperature, the RE was significantly decreased. The solubility of naphthalene increased with temperature, therefore the bioavailability of naphthalene was enhanced and catalyzed the biodegradation process (Lin et al., 2014). However, at very high temperature, the activity of the enzyme reduced due to denature of the enzyme, thus diminishing the effectiveness of the biodegradation process (Geed et al., 2017). In addition, high-temperature process reduces the DO, and subsequently impede the rate of aerobic degradation. The naphthalene RE was decreased from 95 to 87% as the concentration was increased from 10 to 30 mg/L. Lu et al. (2011) have studied the naphthalene biodegradation at various concentrations (1-30 mg/L) using *Pseudomonas* sp. and reported that the RE was sharply reduced due to substrate inhibition to 20.2 % at the maximum concentration of naphthalene. However, the present work shows improved RE as compared to the result obtained by Lu et al. (2011).

3.3.4.3. Effect of pH and temperature

The pH and temperature are acknowledged as the vital parameters that significantly affect the biodegradation of pollutants (Geed et al., 207; Lu et al., 2011). The surface and contour plots of the combined effect of temperature and pH is shown in **Figure 3.6e** and **3.6f**. The naphthalene RE was high at neutral pH. As we proceed towards acidic or alkaline conditions with varying temperature, the RE was significantly decreased. It can be observed from the surface and contour plots, the RE of naphthalene was increased with increase in temperature up to 32.5 °C, whereas the RE was decreased with further increase in temperature. The more acidic or alkaline pH of the solution adversely affects the enzymatic activity of the microorganism, and subsequently impede the biodegradation rate (Chen et al., 2006; Geed et al., 2017). EI-Naas et al. (2014) have reported that the carbon dioxide produced during aerobic biodegradation, which leads to lower the pH of the system and inhibit the rate of biodegradation. The optimal growth of microorganisms was achieved between pH 6.0 to 8.0 (Chen et al., 2006). Thus, a specific range of pH and temperature must be maintained in biodegradation to achieve the effective growth of microorganisms and biodegradation of pollutants.

3.3.5. Model validation

The optimal conditions for the maximum RE of naphthalene were accomplished using a quadratic model within the experimental range of various process variables. The process variables namely; naphthalene concentration, pH, and temperature were predicted via optimization by CCD of RSM and the optimum values were found to be 10.3 mg/L, 6.87, and 31.6 °C, respectively for the maximum RE (97.8%) of naphthalene. In the validation experiment, naphthalene concentration, pH, and temperature were rounded off to 10 mg/L, 7.0, and 32 °C, respectively. The corresponding experimental value of the naphthalene RE was obtained as 96.1%, which is very near to the value predicted by the model.

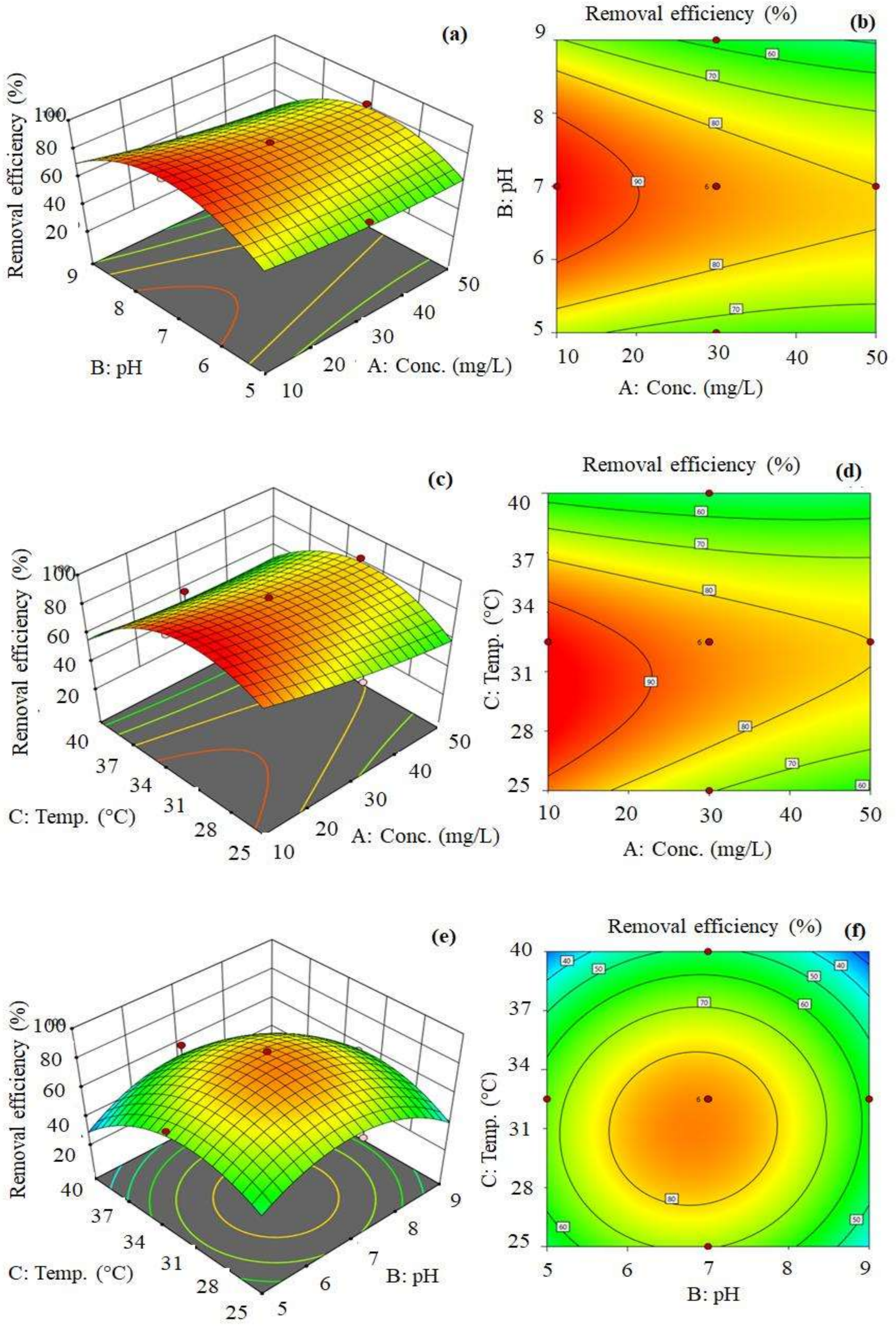

Figure 3.6. Surface and contour plots of naphthalene removal: (a, b) effect of pH and naphthalene conc. (at 32.5 °C temperature); (c, d) effect of temperature and naphthalene conc. (at 7.0 pH); (e, f) effect of pH and temperature (at 30 mg/L conc.).

3.3.6. Performance of laboratory-scale IATP

The performance of IATP was evaluated at different flow rates and ILR under optimum process conditions. The flow rates of wastewater were varied in the range of 200-1000 mL/h. The residual naphthalene concentration was monitored at the regular time intervals. The biodegradation study in IATP was performed at the temperature of 32±3 °C. The summary of the performance of IATP has been given in **Table 3.4**. Initially, the aerobic reactor was operated at an inlet flow rate (IFR) of 200 mL/h. The maximum RE and EC of 91% and 5.26 mg/L/day, respectively were observed until 15th day (**Figure 3.7**). Further, with an increase in the IFR from 200-400 mL/h, the corresponding EC was increased up to 9.98 mg/L/day. The performance of the aerobic reactor recovered quickly and became nearly constant with 86.6 % of RE on the 23rd day of operation. After observing nearly constant RE on 23rd day, the flow rate was enhanced to 600 mL/h on the 24th day of operation. Similarly, on the 33rd and 42nd days, the flow rates of wastewater were enhanced to 800 and 1000 mL/h, respectively. The RE of 74.6% was obtained at 1000 mL/h with the ILR of 28.8 mg/L/day. It was found that the RE was decreased with an increase in the loading rate of influent.

At high flow rate or ILR, the decline in the RE may be due to the short residence time of wastewater inside the bioreactor (Geed et al., 2017). The similar study has been performed by previous researchers and observed that as the inlet flow rate of wastewater increased, the RE was decreased due to the short residence time of reacting fluid inside the bioreactor (Banerjee and Ghoshal, 2017; Dizge and Tansel, 2010; Tepe and Dursun, 2008). Banerjee and Ghoshal (2017) studied the phenol degradation by calcium-alginate immobilized *Bacillus cereus* and found that the biodegradation rate was reduced with an increase in the IFR. Similar kind of results has been reported by previous researchers (Kureel et al., 2017; Srivastva et al., 2015). The pH and DO were varied in the range of 6.2-7.6 and 3.7-6.5 mg/L, respectively. According to Geed et al. (2017), the DO level of the reacting fluid was maintained at high (2.14-5.52 mg/L) to enhance the performance of bioreactor for the biodegradation of pesticide. The similar effects of DO on the removal of substrate were reported by previous researchers (Sonwani et al., 2019b; Yadav et al., 2015). Generally, neutral pH is most favorable for the effective enzymatic activity of bacterial species toward degradation of the organic pollutants (Agrawal and Shahi, 2017; Mudliar et al., 2010).

Table 3.4 Effect of process flow rate, and Inlet loading rate (ILR) on the performance of pilot-scale integrated aerobic treatment plant (IATP).

Flow rate (mL/h)	Time (days)	ILR[a] (mg/L/day)	EC[b] (mg/l/day)	% RE[c]
200	1-15	5.760	0-5.2	0-91.4
400	16-23	11.52	8.6-9.9	74.3-86.6
600	24-32	17.28	11.5-14.1	67.1-81.8
800	33-41	23.04	14.5-18.3	62.9-79.4
1000	42-50	28.80	15.9-21.5	55.4-74.6

[a]Inlet loading rate; [b]Elimination capacity; [c]Removal efficiency

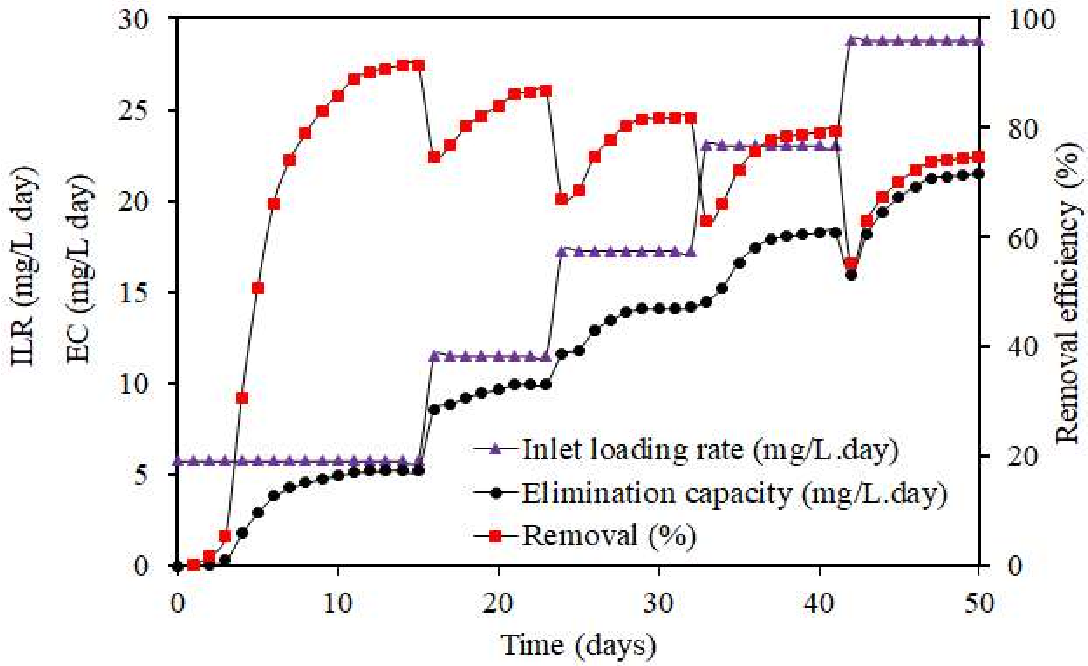

Figure 3.7. The performance of pilot-scale integrated aerobic treatment plant (IATP) for the treatment of naphthalene (ILR: Inlet loading rate, EC: Elimination capacity).

3.3.7. Analysis of metabolites by GC-MS

The biodegradation of naphthalene is a convoluted process due to the involvement of different microorganisms and their diverse metabolic pathways along with the production of a large number of intermediates. In the case of control sample, the standard peak of naphthalene ($C_{10}H_8$) was observed at 15 min (**Fig. 3.8a**). The chromatogram of treated samples shows several peaks corresponding to biodegraded products of naphthalene by *Bacillus cereus*. Among them, catechol ($C_6H_6O_2$) and 2-naphthol ($C_{10}H_8O$) were the major metabolites obtained at 16 min and 24.9 min, respectively (**Fig. 3.8b**). Catechol and 2-naphthol were the common intermediate metabolites observed during biological treatment of naphthalene (Zeinali et al., 2008).

Microorganism favors the aerobic condition for the biodegradation of naphthalene via monooxygenase or dioxygenase enzymes. According to Ghosal et al. (2016), the hydroxylation of naphthalene via dioxygenase formed the diol intermediate, which subsequently transformed into catechol through an ortho or meta cleavage pathway and finally converted to TCA (tricarboxylic acid) intermediates. Similarly, Nievas et al. (2006) reported that monooxygenase and dioxygenase enzymes play a vital role in the degradation PAHs. Zeinali et al. (2008) reported that catechol was the major metabolites formed after degradation of naphthalene using *N. otitidiscaviarum* strain TSH1. These metabolites during the biodegradation of naphthalene by *Bacillus cereus* confirm that naphthalene was biodegraded and obtained intermediates were comparable with the previous reports (Hadibarata et al., 2012; Lin et al., 2010; Zeinali et al., 2008).

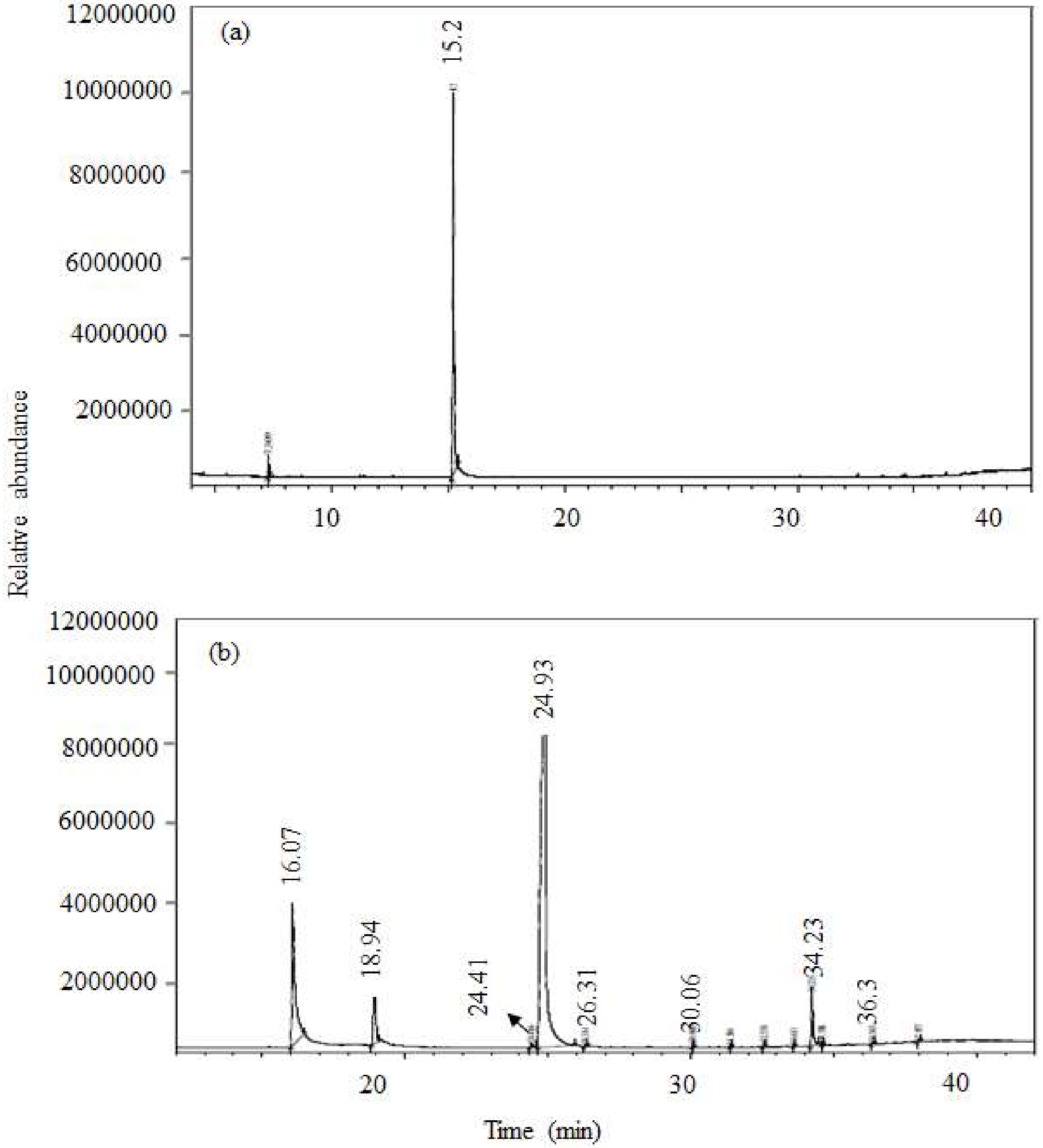

Figure 3.8. GC–MS profile (a) naphthalene control and (b) intermediate metabolites identified in naphthalene biodegradation.

3.3.8. Kinetic study

The kinetics of naphthalene biodegradation have been studied under non-inhibition and substrate inhibition condition using Monod and Teissier-Edwards models, respectively. The profile of experimental data and predicted models have been presented in **Figure 3.9**. The obtained regression values (R^2) more than 0.95 and root mean square error (RMSE) less than 0.01 indicate that the Monod and Teissier-Edwards models were well fitted with experimental data (**Table 3.5**). Based on the Monod growth model, the values of μ_{max} (0.165 per day) and K_s (7.9 mg/L) were obtained (**Figure 3.9a**). The ratio of μ_{max}/K_s is known as specific affinity and used as the convenient index to show the efficacy of microbes in the biodegradation of substrate (Kureel et al., 2017). In this study, the value of μ_{max}/K_s was observed to be 0.021 L/mg.day using Monod growth model.

Teissier-Edwards model was employed to study the kinetics of naphthalene biodegradation under substrate inhibition. It can be observed in **Figure 3.9b** that the value of μ was increased with increasing the concentration of naphthalene up to 30 mg/L and further increased in concentration of naphthalene, and the value of μ was decreased. The decreased in the value of μ may be due to the possibility of substrate inhibition beyond 30 mg/L. The similar effect of substrate on specific growth rate (μ) has been observed by the previous researcher for different substrate (Geed et al., 2017; Kureel et al., 2017; Lu et al., 2011). The values of μ_{max}, K_s, and K_i were obtained to be 0.321 per day, 11.5 mg/L, and 33.2 mg/L, respectively, using Teissier-Edwards model. The obtained value of K_i represents the concentration of naphthalene at which inhibition was observed in the biodegradation. Knightes and Peters (2006) studied biodegradation the kinetic of naphthalene with the concentration of 3.1-10 mg/L and reported that the value of μ_{max}, K_s, and μ_{max}/K_s was found to be 0.64 per hour, 0.57 mg/L and 1.1 L/mg.h, respectively. A summary of kinetics constants obtained for naphthalene biodegradation by the previous researchers has been given in **Table 3.6**. The kinetic results in the present work cannot be compared directly with the previous data due to different substrates concentration and operating parameters.

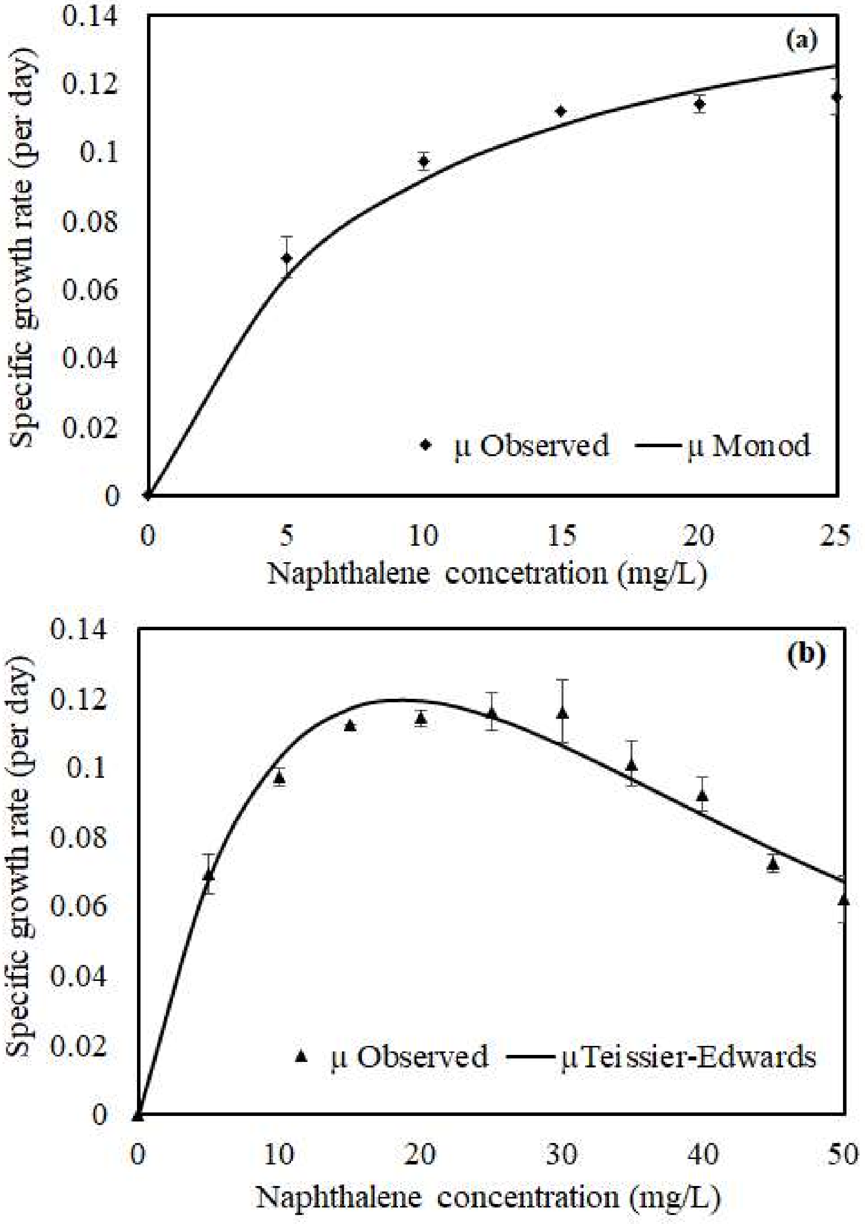

Figure 3.9. Experimental and predicted plots at different naphthalene concentrations: (a) Monod growth model; (b) Teissier-Edwards inhibition model.

Table 3.5 Monod growth kinetics and Teissier-Edwards inhibition model.

Naphthalene Conc. (mg/L)	Model	μ_{max}[a] (per day)	K_s[b] (mg/L)	K_i[c] (mg/L)	μ_{max}/K_s (L/mg.d)	R^2	RMSE[d]
05-25	Monod	0.165	7.91	-	0.021	0.97	0.008
05-50	Teissier-Edwards	0.321	11.5	33.2	0.027	0.97	0.005

[a] Specific growth rate; [b] Half-saturation rate constant; [c] Substrate inhibition constant; [d] Root mean square error

Table 3.6 A summary of kinetics constants obtained for naphthalene biodegradation.

Naphthalene Conc. (mg/L)	Microbe	Model	μ_{max}^{a} (1/h)	K_s^{b} (mg/L)	K_i^{c} (mg/L)	μ_{max}/K_s (L/mg.h)	R^2	References
3-10	*Sphingomonas yanoikuyae*	Monod	0.64	0.57	-	1.12	-	Knightes and Peters (2006)
0.7	*Sphingomonas paucimobilis*	Monod	0.1	0.08	-	1.25	-	Desai et al. (2008)
0-150	Consortium	Monod	0.023	0.075	-	0.31	-	Maillacheruvu and Pathan (2009)
20-100	*Micrococcus sp.*	Monod	0.56	21.4	-	0.026	0.99	Jegan et al.(2010)
10-100	*Streptomyces* sp.	Andrews	1.56	81.76	60.3	0.026	0.97	Xu et al. (2014)
10-100	*Chryseobacterium Arthrosphaerae* & *Rhodobacter maris*	Monod	0.4	10	-	0.04	-	Oberoi et al. (2015)
5-50	*Exiguobacterium sp.*	Andrews-Haldane	0.016	13.6	20.5	0.001	0.96	Sonwani et al. (2020b)
5-25	*Bacillus cereus*	Monod	0.007	7.91	-	0.001	0.97	This study
5-50		Teissier-Edwards	0.013	11.5	33.2	0.001	0.97	

3.3.9. Phytotoxicity analysis

The *Cicer arietinum* seeds were germinated in distilled water (control), untreated and treated wastewater is shown in **Figure 3.10**. The seeds germinated in untreated wastewater shows 73% germination, 1.21 ± 0.13 cm of average root length, and 0.51 ± 0.11 cm of average shoot length, whereas seeds grown in treated wastewater shows 93% germination, 3.02 ± 0.28 cm of average root length, and 3.02 ± 0.28 cm of average shoot length (**Table 3.7**). The seeds grown in distilled water indicates 100% of germination, 3.5 ± 0.10 cm of average root length, and 3.19 ± 0.12 cm of average shoot length. It can be observed that when seed germinated in treated wastewater shows better growth in terms of germination, shoot, and root length than untreated wastewater. Also, the seeds germination in treated wastewater is more closely to distilled water.

Similar kinds of outcomes were observed in previous studies for the biodegradation of different pollutants (Kumar et al., 2019; Sutar et al., 2019). Kumar et al. (2019) have studied the toxicity effect of treated and un-treated Acid black 24 dye on *Vigna radiata* and *Sorghum vulgare* seeds. They reported that when *Vigna radiata* seeds germinated in untreated Acid black 24 revealed about 2.3 times less germination than the treated solutions. Similarly, *Sorghum vulgare* seeds subjected to the untreated dye shown about 2.2 times less germination than the treated solution. The improved seeds growth in treated wastewater indicates that the treated wastewater could be used for irrigation purpose. However, some more analysis is required prior to its application in real irrigation purpose.

Table 3.7 Summary of phytotoxic analysis of the treated and untreated wastewater on *Cicer arietinum* seeds.

S.N.	Parameter	Distilled water	Untreated wastewater	Treated wastewater
1	Germination (%)	100	73	93
2	Root length (cm)	3.5 ± 0.10	1.21 ± 0.13	3.02 ± 0.28
3	Shoot length (cm)	3.19 ± 0.12	0.51 ± 0.11	2.75 ± 0.24

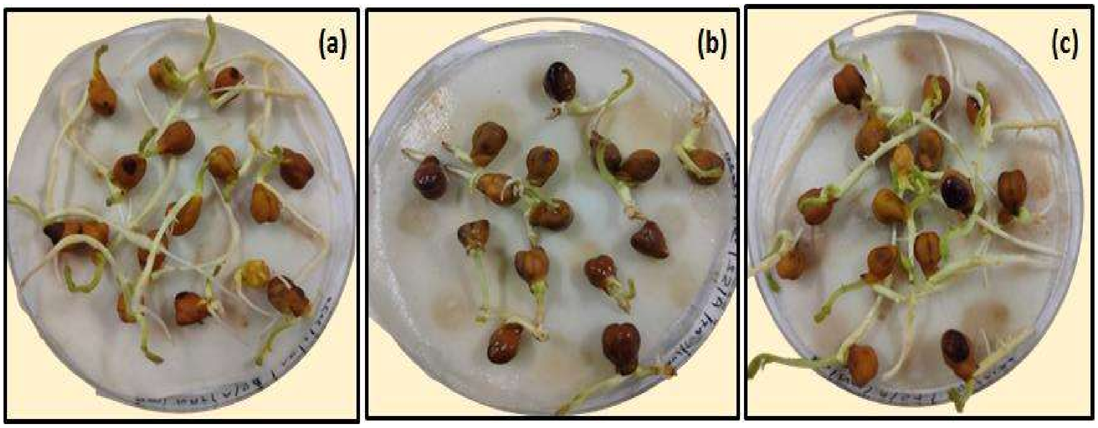

Figure 3.10. Images of *Cicer arietinum* seeds germinated in (a) distilled water, (b) untreated wastewater, and (c) treated wastewater.

3.4. Conclusions

Initially, ten bacterial species (RKS1, RKS2, RKS3, RKS4, RKS5, RKS6, RKS7, RKS8, RKS9, and RKS10) were isolated from petroleum-contaminated soil. Amongst them, *Bacillus cereus* RKS4 (MH681588.1) was the most potential bacterial species and successfully used in the biodegradation of naphthalene. The CCD of RSM has been used to achieve the optimal working conditions for the naphthalene biodegradation with least number of experiments. The maximum removal of naphthalene (96.1%) were obtained at optimum conditions (pH of 7.0, naphthalene concentration of 10 mg/L, and temperature of 32.0 ◦C). Further, to scale up the biodegradation process, the performance of a lab-scale IATP in terms of removal efficiency (RE), inlet loading rate (ILR), and elimination capacity (EC) was studied and found that the RE was significantly high at a low loading rate. The kinetic study by Monod and Teissier-Edwards reveals that the substrate inhibition was perceived at high concentration naphthalene. The improved growth of *Cicer arietinum* seeds in treated wastewater indicates that the treated wastewater could be used for irrigation purpose. So, the