Chapter III:

MATERIALS AND METHODS

3.1 Chemicals and media

3.1.1 Chemicals

Sodium arsenite ($NaAsO_2$; HPLC Lab Reagents, 98%) and Sodium arsenate heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$; LobaChemie, 98%) were used for the preparation of stock solutions of As(III) and As(V). Potassium permanganate ($KMnO_4$; Finar, >99%) and potassium hydroxide (KOH; Merck, >85%) were used for modification of the biochar. Sodium hydroxide (NaOH; Merck, >97%) was used for the regeneration study of the biochar. PVA ($[CH_2\text{-}CHOH]_n$, Mw: 70,000 to 100,000, HIMEDIA), SA (S. D. Fine-Chem Limited, Mumbai, India) and Calcium chloride ($CaCl_2$, >98%, Merck) were used for the preparation of PVA/SA beads for encapsulation of immobilized bacteria. Chromium trioxide (CrO_3; Qualigens, 98.5%) was used to prepare a stock solution of Cr(VI). All reagents used in the research work were of the analytical grade, and glassware was cleaned with an HNO_3 solution followed by rinsing with distilled water to prevent any type of contamination.

3.1.2 Media

3.1.2.1 Luria Bertani media

Luria Bertani (LB) media is a versatile and widely used growth medium for the cultivation of bacteria. It is also known as Lysogeny Broth and was developed by Salvador Luria and Giuseppe Bertani. The culture medium (LB) has the following composition per liter of distilled water: tryptone (10 g), NaCl (10 g), and yeast extract (5 g).

3.1.2.2 Luria Bertani agar media

In addition to the components of LB broth, LB agar media contains agar as a solidifying agent. This media was used in the present study for obtaining pure isolates of the bacteria and storing bacteria on plates. 1.5 g to 2.0 g of agar was added to every 100 mL LB broth. Very low concentration of agar results in delay in solidification when poured on plates, and very high concentration can result in solidification even before media is poured on plates. Hence, an optimum amount of agar is required to be added to the broth for preparation of solid media.

3.2 Analytical techniques and instruments

3.2.1 Determination of arsenic

3.2.1.1 Molybdenum blue method

Arsenic concentration was determined by the molybdenum blue (MB) method as defined by Sattar et al. (2019). One mL ascorbic acid solution and 2 mL of 'Reagent A' solution was successively added to a 40 mL sample and the volume was then made to 50 mL using

deionized water. A blank sample was also prepared in the same way by using an appropriate amount of deionized water. The mixture was then left for 60 min at room temperature for complex formation and color development. The absorbance was measured at 861 nm (wavelength was selected by scanning the spectra for maximum absorbance for the spectrophotometer) using SL 159 UV–Vis Spectrophotometer. Reagent A was prepared by dissolving two separate 100 mL solutions of 13 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and 0.35 g of $K(SbO)C_4H_4O_6{\cdot}H_2O$ with a solution of 9M H_2SO_4 in a 500 mL volumetric flask. Ascorbic acid solution was prepared daily by dissolving 10 g ascorbic acid in 100 mL of deionized water before use (Sattar et al., 2019). Concentration of As(III) was determined by oxidizing solution with $FeCl_3$.

3.2.1.2 Arsenator

Arsenic analysis was performed using a digital arsenator (Wagtech - WE10500). Arsenic removal filter papers, placed into the appropriate filter slides (red and black), were inserted into trifilter arsenic trap (also known as bung device) along with hydrogen sulfide removal filter at the bottom. The arsenator was calibrated by placing the filter paper from the black filter slide into the slot and starting the timer. A 50 mL sample was pipetted into a conical flask. Sachet containing A1 powder was added to the sample and stirred for 2 - 3 min, followed by addition of A2 tablets. The loaded bung device was used to seal the flask and prevent leakage of arsine gas. Following this, the flask was left undisturbed for 20 min. Afterward, the mercuric strip in the black filter slide changed color, and it was then placed in the arsenator to analyze the arsenic concentration in the solution.

3.2.2 Determination of chromium

3.2.2.1 Diphenylcarbazide method

The filtered sample (filtered through a 0.45 µm membrane filter) was pipetted into a 100 mL beaker. pH of the solution was adjusted to 1.0 ± 0.3 using 0.2N H_2SO_4. The sample was then transferred into a 100 mL volumetric flask and 2.0 mL of diphenylcarbazide solution [prepared by dissolving 250 mg of 1,5-diphenylcarbazide (1,5-diphenylcarbohydrazide) in 50 ml acetone] was added to it. It was then diluted to 100 mL with distilled water, thoroughly mixed and allowed to stand for 5 to 10 min. A blank solution was also prepared in a similar manner using distilled water as a sample. Absorbance was measured at 540 nm, using a reagent blank as a reference solution (IS 3025 (Part 52), 2003; Standard Methods, 2017).

3.2.3 UV-visible spectrophotometry

UV-visible spectrophotometry works on the principle of Beer-Lambert Law, which states that there is a linear relationship between the absorbance of a solution and its concentration, path length, and the molar absorptivity of the analyte at a specific wavelength. In UV-visible spectroscopy, when a molecule absorbs UV or visible light, it undergoes electronic transitions, where electrons are excited from a ground state to higher energy levels. This absorption of light corresponds to the energy required to promote electrons to higher energy levels, often associated with specific molecular orbitals. The resulting spectrum reveals peaks at wavelengths where absorption occurs. The concentration of the analyte in a sample can be determined by comparing the absorbance of the sample at a particular wavelength to a calibration curve created from known standards (Sawyer et al., 2003).

3.2.4 Preparation of calibration curve

3.2.4.1 Arsenic

Stock solution of 1.0 mg/L As(V) was prepared by using $Na_2HAsO_4.7H_2O$. Standard solutions ranging from 10 µg/L to 1.0 mg/L were prepared using the stock solution. One mL ascorbic acid solution and 2 mL of 'Reagent A' solution was successively added to a 40 mL sample and the volume was then made to 50 mL using deionized water. Blank sample was also prepared in the same way by using an appropriate amount of deionized water. The mixture was then left for 60 min at room temperature for complex formation and color development. The absorbance was measured at 861 nm using spectrophotometer. Calibration curve was plotted between absorbance values and arsenic concentration (Figure 3.1).

3.2.4.2 Chromium

Stock solution of 1.0 mg/L Cr(VI) was prepared by using CrO_3. Standard solutions ranging from 100 µg/L to 1.0 mg/L was prepared using the stock solution. Measured volumes of standard chromium solution were pipetted out into 100 mL beakers and volume was made up to 50 mL with distilled water to obtain varying concentrations for standard solution. pH was adjusted to 1.0±0.3 with 0.2N H_2SO_4. These solutions were transferred quantitatively into 100 mL volumetric flasks and 2.0 mL of diphenyl carbazide solution was added to each sample. It was then diluted to 100 mL with distilled water, mixed and allowed to stand for 5 to 10 min for full color development. Similarly, a reagent blank was prepared with 10 mL of distilled water. With reagent blank as reference solution, the absorbance of the standard solutions was measured at 540 nm using spectrophotometer. Calibration curve was plotted between absorbance values and chromium concentration (Figure 3.2).

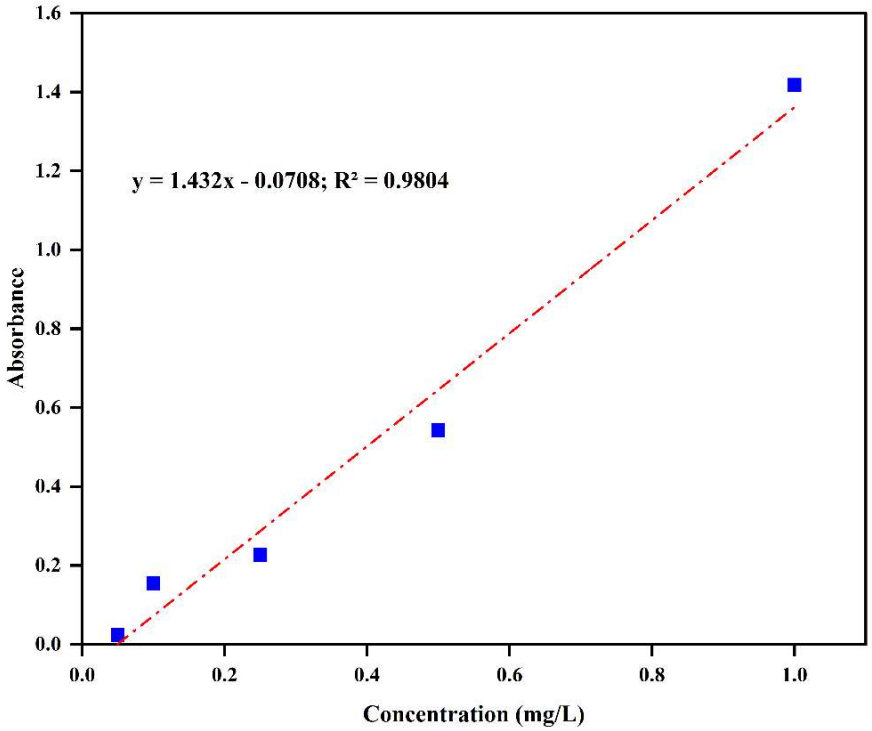

Figure 3.1: Calibration curve for As(V) using MB method

3.2.5 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is a laboratory technique designed to rapidly amplify a specific deoxyribonucleic acid (DNA) segment. The procedure consists of three main steps as outlined below: (i) denaturation (separation of DNA strands), (ii) annealing (attachment of primers to DNA template) and (iii) extension (DNA polymerase synthesizes a complementary strand). These stages are performed in cycles and result in an exponential amplification of DNA. DNA template, primers, DNA polymerase, nucleotides, and a buffer solution are necessary for PCR. DNA analysis, cloning, sequencing and diagnostic applications use PCR extensively because of its ability to selectively amplify target DNA sequences. The 16S rRNA gene, present in all bacteria and featuring highly conserved sequences, is commonly employed for bacterial identification. Certain bacterial species can be recognized by looking at the 16S rRNA nucleotide sequence and cross-referencing it with a database of known sequences.

3.2.6 Characterization

The surface morphology of PSB, MPSB, PUF and beads was observed by scanning electron microscope (SEM) (EVO - Scanning Electron Microscope MA15/18; CARL ZEISS MICROSCOPY LTD.). The composition of elements was determined using energy-dispersive X-ray (EDX) analysis (Rigaku SmartLab 9kW Powder type, RIGAKU

Corporation). Fourier transform infrared spectroscopy (FTIR) (Nicolet iS5; THERMO Electron Scientific Instruments LLC) was used to study the functional groups. The FTIR spectra were observed between wavenumber 400 to 4000 cm^{-1} at 4 cm^{-1} resolution. Specific surface area and pore volume of PSB and MPSB were determined by BrunauerEmmett-Teller (BET) analysis using BELLSORP MAX II & BELCAT-II (MicrotracBEL Corp.) by N_2 adsorption and desorption isotherm technique. The X-ray diffraction (XRD) patterns of PSB and MPSB were recorded with a Rigaku–Ultima IV (XRD) using Cu Kα X-ray lines as the radiation source at 40 kV and 40 mA power in the range of 10–80°.

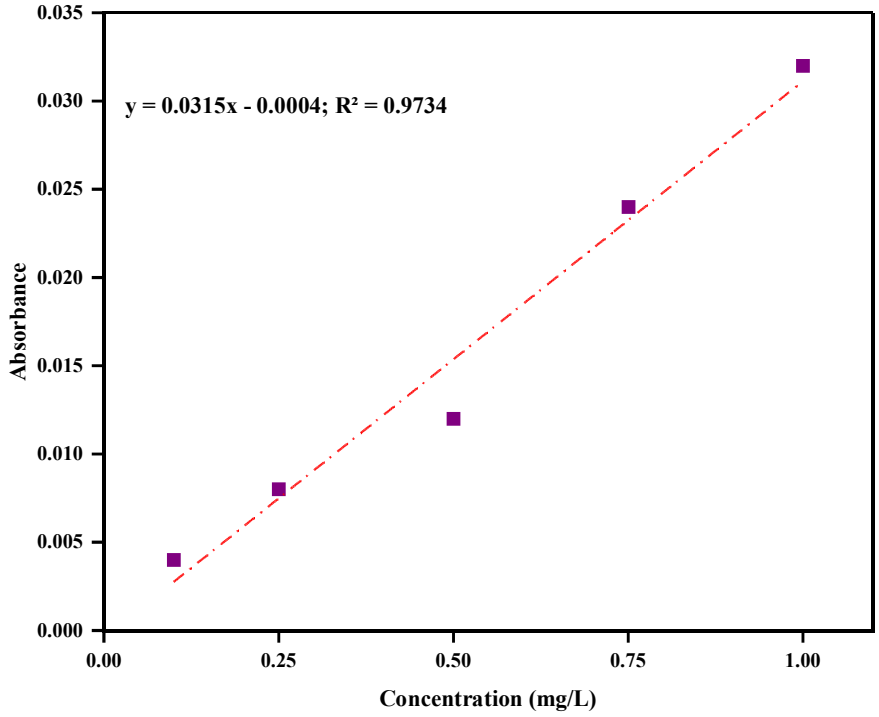

Figure 3.2: Calibration curve for Cr(VI) using diphenylcarbazide method

3.3 Methods

3.3.1 Sample collection

The soil sample was collected from the subsurface region of Ratanpur Village of Varanasi district, Uttar Pradesh, India, which is known to have arsenic contamination (Raju, 2012), and the coordinates of the location were 25°18'45.0288" N and 83°2'29.1624" E (Figure 3.3). The soil was collected in clean polythene bags and was preserved at 4°C for further use. Total arsenic was determined as per the procedure described by Banerjee et al. (2011). In brief, 2.5 g of soil was digested in aqua regia [HCl:HNO_3 (3:1)], heated at 70°C for 1 h and then diluted to 5 mL using distilled water. The sample was filtered and arsenic content was determined.

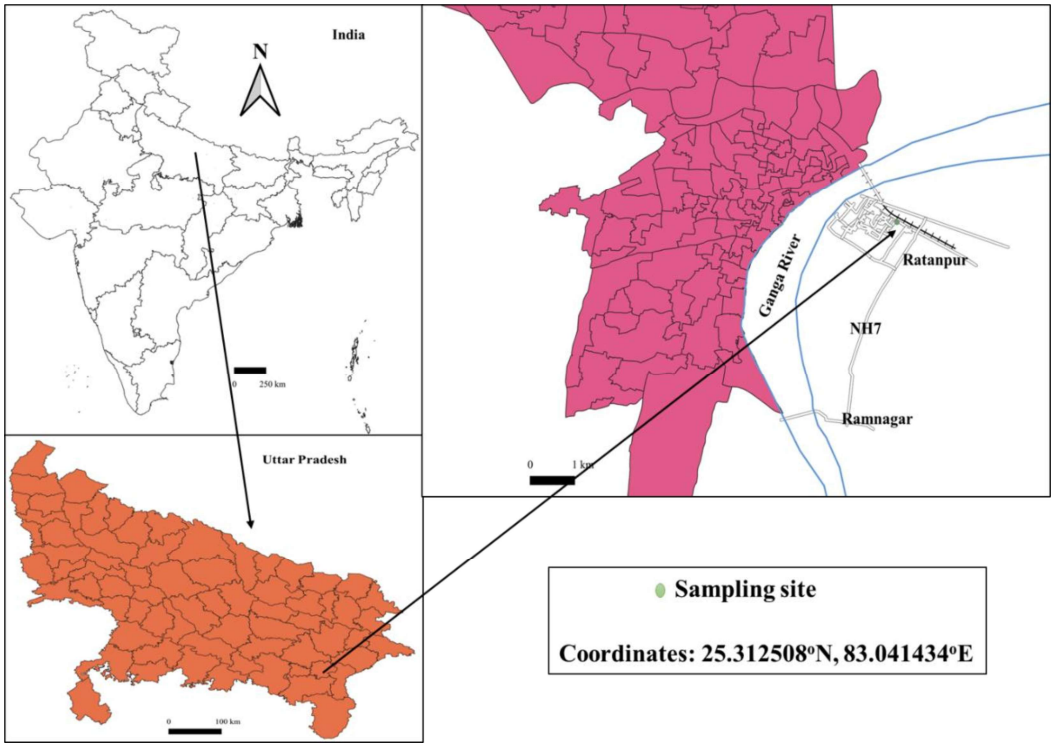

Figure 3.3: Location of sampling site

3.3.2 Enrichment and isolation of arsenic-resistant bacteria

One gram of soil sample was added to 9 mL of distilled water which then serially diluted five to six times. The sample taken from diluted solution (five times) was inoculated in LB media supplemented with 0.5 mM of arsenic and incubated for 15 days for acclimatization. Later, samples were taken from inoculated media and were added to LB media supplemented with 1 mM As(III) and 1 mM As(V), separately and incubated (Model - NSW 256, Brand – Caltan, Country - India) at 30°C for 48 h at 100 rpm. The cultures were enriched by transferring 1 mL of the already cultured bacteria to LB media supplemented with 5 mM of As(III) and As(V), separately and incubated at 30°C for 48 h at 100 rpm. The enriched culture was then streaked on solidified LB media amended with As(III) and As(V), separately and pure culture was obtained by successive culturing of the bacteria three times. A step-wise approach for serial dilution and streaking on agar plates for isolation of bacteria has been shown in Figure 3.4.

3.3.3 Identification of the isolated bacterial strains and phylogenetic analysis

From isolated species inoculated in LB media, DNA was extracted for molecular characterization. 16S rRNA of the extracted DNA was amplified by subjecting it to PCR using universal primers 243F (5′-GGATGAGCCCGCGGCCTA-3′) and 1378R (3'-

CGGTGTGTACAAGGCCCGG-5'). The sequencing of isolated species was carried out by Triyat Scientific Pvt Ltd, Nagpur, India. The PCR was performed using the following thermal cycling conditions: initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min (Jaiswal et al., 2023). The sequences were aligned against the available sequences in the online National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree was constructed using the weighbor method (weighted version of Neighbor Joining) in Molecular Evolutionary Genetics Analysis (MEGA) (version 10.2.5; Pennsylvania State University) software. The accession numbers for the isolates were obtained by depositing the sequences to NCBI Genebank.

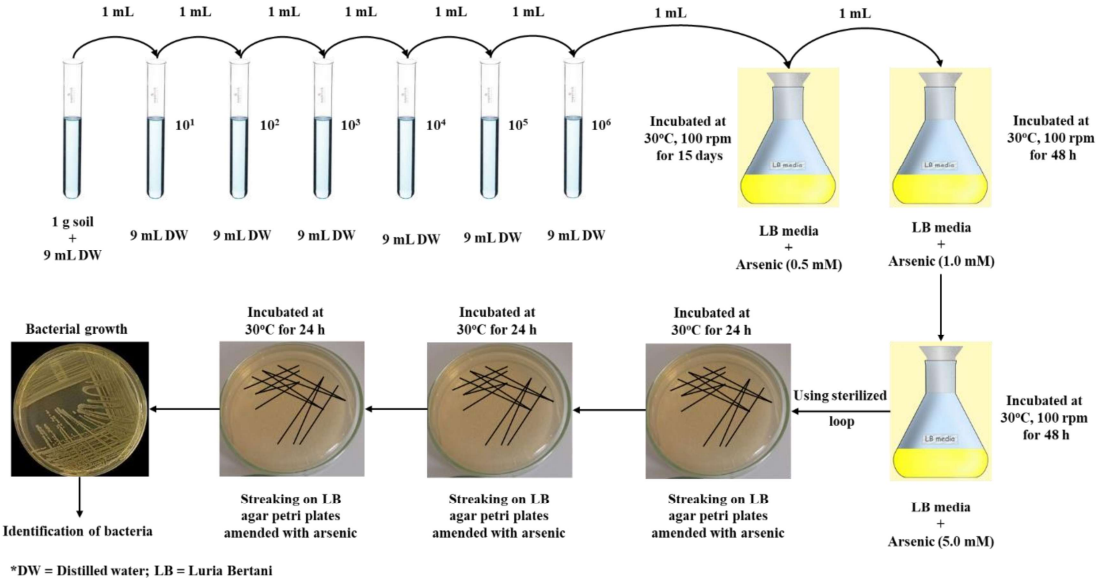

Figure 3.4: Serial dilution and streaking on agar plates for isolation of bacteria

3.3.4 Determination of minimum inhibitory concentration

In this study, the growth of isolated bacteria at different concentrations of arsenic was observed to determine the Minimum Inhibitory Concentration (MIC) of the selected isolates for As(III) and As(V) (Aguilar et al., 2020). Freshly grown bacteria were added to LB media supplemented with varying concentrations of As(III), from 1 to 50 mM, (0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mM) and of As(V) (1, 10, 50 100, 200, 400 600, 800, 1000 mM), separately and were incubated at 30°C for 24 h at 100 rpm. LB media was seeded with bacteria without arsenic to be used as control. Optical density (used as an indicator for bacterial growth) was measured at 600 nm using spectrophotometer (Pandey and Bhatt, 2015).

3.3.5 Parameter optimization for bacterial growth

3.3.5.1 Effect of pH and temperature

To optimize pH for bacterial growth, LB media was seeded with the isolated strain in the presence of 1 mM As(III) and 1 mM As(V) respectively, in separate flasks and pH was varied from 3 to 12 (Roychowdhury et al., 2018) and the cultures were incubated at 30°C for 24 h at 100 rpm. Various combinations of acid and base were used to maintain pH and optimization was performed in three cases. In case 1 - HCl and KOH; in case 2 - H_2SO_4 and NaOH; and in case 3 - HNO_3 and KOH were used to maintain pH. For temperature optimization, LB media was inoculated with isolated bacterial strain in the presence of 1 mM As(III) and 1 mM As(V) respectively, in separate flasks and was incubated at temperatures varying from 20°C to 50°C (20, 25, 30, 35, 40, 45, 50°C) for 24 h at 100 rpm. Bacterial growth was measured at 600 nm using a spectrophotometer.

3.3.5.2 Effect of initial arsenic concentration and inoculum dose

LB media was supplemented with As(III) and As(V) in varying concentrations, ranging from 1 mM to 10 mM. Another LB media was inoculated with bacteria without the presence of As(III) and As(V) to be used as control. The cultures were then incubated at 30°C for 24 h at 100 rpm. To study the effect of inoculum dose, LB media, supplemented with arsenic, was inoculated with varying volumes (0.5, 1.0, 1.5, 2.0, 2.5 mL) of already cultured bacteria. The volume of LB media was kept constant and inoculated media was incubated at 30°C for 24 h at 100 rpm. Bacterial growth was then measured at 600 nm using a spectrophotometer.

3.3.6 Determination of biotransformation ability of bacteria

The biotransformation ability of the isolated strains was determined using the $AgNO_3$ test (Krumova et al., 2008). Isolated strains were cultured separately on agar media amended with 500 µg/L of As(III) and As(V). After incubation, plates were flooded with 0.1M $AgNO_3$. The formation of a brownish precipitate indicates the presence of arsenate [As(III) oxidizing bacteria] and a yellow precipitate indicates the presence of arsenite [As(V) reducing bacteria]. To study the effect of $AgNO_3$ with time, eight different combinations of arsenic and bacteria (1 – As(III)_K7Pb; 2 - As(V)_TY6; 3 – As(III)+As(V)_K7Pb; 4 - As(III)+As(V)_TY6; 5 - As(III)_TY6; 6 - As(V)_K7Pb; 7 - As(V)_K7Pb+TY6; 8 - As(III)_K7Pb+TY6) were used (***Note**: K7Pb and TY6 refer to the name of isolated bacterial strains*). The biotransformation ability was also confirmed with the $KMnO_4$ test as described by Salmassi et al. (2010). 40 µL of 0.01M $KMnO_4$ solution was added to 2 mL of sample. The formation of pink color indicated the presence of arsenate (does not react

with permanganate) and the formation of orange color indicated the presence of arsenite (reacts with permanganate).

3.3.7 Resistance of bacteria against other heavy metal(loid)s

Ability of the isolated bacteria to tolerate heavy metal(loid)s other than arsenic, such as cadmium (Cd) ($CdCl_2.H_2O$, Lobachemie, 98%), chromium (Cr) ($CrCl_3.6H_2O$, Lobachemie, 93%), cobalt (Co) ($CoCl_2.6H_2O$, Lobachemie, 98%), lead (Pb) (PbO_2, Lobachemie, 95%), mercury (Hg) ($HgBr_2$, Merck, 99%) and nickel (Ni) ($NiSO_4.6H_2O$, Merck, 99%), was evaluated at different concentrations. A fresh culture of bacteria was added to LB media supplemented with varying concentrations of different metal(loid)s. Cultures were incubated at 30°C for 72 h at 100 rpm. Bacterial growth was then measured at 600 nm using a spectrophotometer and MIC for different elements was determined.

3.3.8 Phytotoxicity assessment

Phytotoxicity assessment for untreated and treated water was done using seeds of *Vigna radiata* and chickpea. Seeds were purchased from a local market near IIT (BHU) Varanasi, India. Healthy and equal-sized seeds (10 numbers of each, *Vigna radiata* and chickpea) were selected and sterilized with 10% (w/w) solution of hydrogen peroxide for 10 min and were washed thrice with distilled water to avoid any fungal infections (Cao et al., 2007). Different combinations of treated and untreated samples were used for the experiment. Untreated solutions containing 500 µg/L arsenic [As(III) and As(V)] and solutions treated with TY6, K7Pb and TY6+K7Pb were used and distilled water served as control. The selected seeds were kept on pre-wetted filter paper in petri plates at room temperature and irrigated regularly. Their growth and germination were monitored for 7 days. The seeds were supposed to have been germinated when the root length (RL) was 2 mm (Balestri et al., 2019). Germination (%) was calculated based on 2 days of observation and other parameters such as relative root growth (%RRG), relative seed germination (%RSG), phytotoxicity index (PI) and germination index (%GI) were calculated based on 7 days of observation as per the equations (3.1), (3.2), (3.3) and (3.4), respectively (Luo et al., 2018).

$$\%RRG = \frac{root\ length\ of\ sample}{root\ length\ of\ control\ sample} \times 100 \quad \text{---- (3.1)}$$

$$\%RSG = \frac{germination\ of\ seed\ in\ sample}{germination\ of\ seed\ in\ control\ sample} \times 100 \quad \text{---- (3.2)}$$

$$PI = 1 - \frac{root\ lenth\ of\ sample}{root\ length\ of\ control\ sample} \quad \text{---- (3.3)}$$

$$\%GI = \frac{RRG \times RSG}{100} \quad \text{---- (3.4)}$$

3.3.9 Recirculating packed bed bioreactor

The set up for recirculating packed bed bioreactor (RPBBR) has been shown in Figure 3.5. The reactor was made of borosilicate glass with dimensions of 60 cm height and 5.5 cm inner diameter. The working volume of the reactor was approximately 1.2 L. PUFs of cubic shape with dimensions 1 cm x 1 cm x 1 cm were sterilized and used as packing material. The influent was fed through a peristaltic pump (ELECTROLAB, PP- 50 V) from the feed tank and the flow rate was varied using a rotameter (Flow Point, India). Samples for analysis of arsenic concentration were collected on a daily basis from the sampling point at the top.

3.3.10 Immobilization of bacteria and operating conditions of RPBBR

The reactor was run for 20 days in batch mode with LB Broth media, inoculated with TY6 and K7Pb, in separate reactors, for immobilization of bacteria. The reactor was operated at two different flow rates of 5 mL/min and 10 mL/min. The initial concentration of As(III) and As(V) was varied from 250 µg/L to 50 mg/L. pH of the influent media was kept the same as was obtained during the optimization process in the batch studies. When the arsenic concentration reached below the permissible limit, or there was no further removal, the feed tank and reactor were drained and a fresh sample of arsenic with increased concentration was added to the feed tank. The morphology of PUF before and after immobilization for biofilm formation was analyzed using SEM.

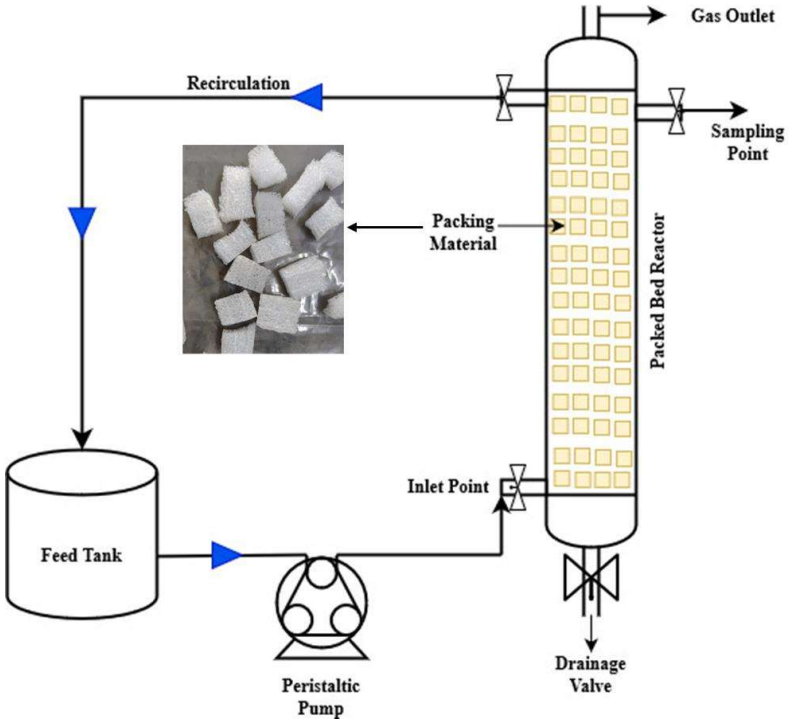

Figure 3.5: Schematic diagram of RPBBR for arsenic bioremediation

3.3.11 Bacterial toxicity assessment

Luminescent toxicity assessment is one of the fastest ways to determine the acute and chronic toxicity effects of bacteria. Various luminescent bacteria such as *Photorhabdus luminescens*, *Vibrio fischeri*, *Pseudomonas fluorescens*, etc. (Menz et al., 2013; Tiwari et al., 2022) have been used for toxicity analysis. Bioluminescence results due to the reduction of flavin mononucleotide (FMN) to flavin mononucleotide ($FMNH_2$) by the action of luciferase enzyme. The reduced $FMNH_2$, along with long-chain aliphatic aldehyde, gets further oxidized in the presence of oxygen to produce light (equation 3.5) (Chaturvedi et al., 2021; Tiwari et al., 2023).

$$FMNH_2 + R - CHO + O_2 \rightarrow FMN + R - COOH + H_2O + Light \quad \text{---- (3.5)}$$

In this work, *Photorhabdus luminescens* subsp akhurstii was used for toxicity assessment. Lyophilized bacteria were obtained from the National Centre for Microbial Resource (NCMR) (Pune, India), which were cultured in LB media and used for further experiments. TY6 and K7Pb were cultured in LB media, with and without arsenic, for the experiments and sterilized distilled water served as control. The samples were then centrifuged at 5000 rpm for 10 min, filtered with 0.44 µm Whatman filter paper and sterilized. One mL bacterial culture of *Photorhabdus luminescens* with optical density of approximately 1 was added to 10 mL of control, treated and untreated samples, which were then incubated for 1 h (acute) and 24 h (chronic). Bioluminescence intensity (as counts per second) was measured using a Horiba Fluorescence Spectrophotometer (PTI QuantaMaster™ 8000 series). Bioluminescence inhibition percentage (BI%) was calculated using equation 3.6:

$$BI\ (\%) = \frac{\text{Control Intensity} - \text{Sample Intensity}}{\text{Control Intensity}} \text{ X } 100\ \% \quad \text{---- (3.6)}$$

3.3.12 Soil sample preparation for the study

Soil sample was collected from an agricultural field of the Banaras Hindu University, Varanasi. The soil was dried, sieved and was rid of any foreign materials before it was used for experiments. Stock solutions of arsenic and chromium were prepared in acetone separately. The prepared solutions were mixed with the soil to obtain concentrations of 5, 10 and 20 mg/kg. The soil was stirred manually for 30 min to obtain a homogenous mixture. Freshly incubated bacteria were then added to the soil. Samples were collected at regular intervals for analysis. Various combinations of arsenic and chromium were used to study the effect of concentration on the removal efficiency of the bacteria. The combinations have been listed in Table 3.1. Apart from the samples listed in Table 3.1, sterilized soil samples

were also prepared without bacteria to observe the effect of abiotic components on the removal of arsenic and chromium.

Table 3.1: Different combinations of soil sample, arsenic, chromium and bacteria

S. No.	Sample name	Meaning	Description
1	Control	Soil sample without bacteria	Prepared separately for As(III), As(V) and Cr(VI) each, designated as A_Control and C_Control, respectively
2	A_5	Arsenic concentration – 5 mg/kg	Prepared separately for As(III)_TY6 and As(V)_ K7Pb
3	A_10	Arsenic concentration – 10 mg/kg	
4	A_20	Arsenic concentration – 20 mg/kg	
5	A + C_5	Arsenic and chromium concentration – 5 mg/kg each	Prepared separately for As(III), As(V) and Cr(VI) with TY6 and K7Pb respectively
6	A + C_10	Arsenic and chromium concentration – 10 mg/kg each	
7	A + C_20	Arsenic and chromium concentration – 20 mg/kg each	

3.3.13 Extraction of arsenic and chromium from soil

After treatment, arsenic and chromium were extracted from the soil sample for determination of concentration. Arsenic was extracted using $CaCl_2$, distilled water (DW) and aqua regia (AR) and chromium was extracted using NaOH, DW and AR. Arsenic was extracted from soil as per the procedure described by Houba et al. (2008). In brief, the dried soil sample was mixed with 0.01 M $CaCl_2$ solution in the ratio of 1:10 (w/v) and was shaken for 2 h. The sample was then filtered through the Whatman 1 filter paper. Later, the filtrate was centrifuged at 5000 rpm for 10 min and filtered again. 0.1 mL of 1 M HCl was added to 10 mL of solution to prevent elemental adsorption on the test tube and the growth of bacteria. The acidified solution was stored at 4^{o}C for determination of arsenic afterwards. The same procedure was followed with DW. Chromium was extracted using steps as described by James et al. (1995). Briefly, 2.5 g of oven-dried soil was added to 50 mL of 0.1 M NaOH solution. The samples were then transferred to a sonicating bath containing distilled water for 45 min and were swirled briefly every 15 min during the duration of sonication. The samples were filtered through Whatman 1 filter paper, centrifuged at 5000 rpm for 10 min and filtered again. Afterwards, samples were stored at 4^{o}C for determination of chromium. The same procedure was followed with DW.

For AR, arsenic extraction was done as per the steps by Banerjee et al. (2011). In brief, 2.5 g of oven-dried sample was mixed with AR ($HCl:HNO_3$ = 3:1), heated to 70°C for 1 h and diluted to 5 mL with deionized water. The digested solution was filtered and arsenic content was determined. For chromium, the steps followed were as described by McGrath and Cunliffe (1985). In brief, 0.5 g soil was mixed with 8 mL HCl and 2 mL HNO_3 and heated to 60°C for 2 h. It was then digested at 105°C for 1 h. The temperature was then increased to 140°C until the samples were dried. After cooling the samples, 12.5 mL of 20% (by volume) HCl was added and the mixture was heated to 80°C for 20 min. The mixture was cooled, filtered and diluted to 50 mL with deionized water.

3.3.14 Extraction of arsenic and chromium from plant

Plants were washed with distilled water thoroughly so that no soil was attached to the roots. The root and shoot were separated and placed in the oven to dry at 60°C until a constant weight was obtained. The dried plants were then ground in ceramic crucibles and sieved to obtain powders of uniform size. The extraction of arsenic from the root and the shoot was performed as per Gulz et al. (2005). In brief, 0.5 g of the sample, root and shoot separately, was digested with 5 mL HNO_3 and 3 mL H_2O_2. Deionized water was used to make the final volume up to 25 mL for analysis. The extraction of Cr(VI) was carried out using a procedure described by Panichev et al. (2005). In brief, 0.25 g of sieved samples (root and shoot separately) were mixed with 25 mL of 0.1 M Na_2CO_3 and boiled for 10 min. The mixture was centrifuged and filtered, and the final volume was made to 25 mL with distilled water and chromium was determined in the samples.

3.3.15 Plant growth and chlorophyll assessment

To assess the effect of bacteria on plant growth, healthy and equal-sized seeds of chickpeas (10 numbers) were selected, sterilized with a 10% solution of hydrogen peroxide and washed with deionized water (3 times) to avoid any fungal infection. The seeds were then planted in the soil sample and were monitored over time. Shoot length was measured on day 5, day 10 and day 15.

Chlorophyll absorbs energy from sunlight and synthesizes CO_2 and carbohydrates through photosynthesis. Two types of chlorophylls are found in higher plants, chlorophyll *a* (blue-green in color) and chlorophyll *b* (yellow-green in color) (Manolopoulou et al., 2016). Chlorophyll *b* absorbs energy and transfers it to chlorophyll *a* and is essential to stabilize proteins responsible for light harvesting (Jaiswal et al., 2023; Kume et al., 2018). Chlorophyll determination was done using the dimethyl sulfoxide (DMSO) method, as described by Manolopoulou et al. (2016). 0.1 g of freshly grown leaves were plucked from

the chickpea plant, cut into small pieces and mixed with 10 mL DMSO. The mixture was then heated to 60 – 70ºC for 1 h and was then followed by cooling for 30 min at room temperature. It was centrifuged at 5000 rpm for 10 min and filtered using Whatman filter paper. The absorbance of the samples were measured using a spectrophotometer with DMSO as blank at 665 nm and 648 nm. Chlorophyll concentration (a, b and total) was expressed as mg/g fresh weight and determined by the equations 3.7, 3.8 and 3.9:

Chlorophyll *a* (mg/g F.W.) = $14.85*A_{665} - 5.14*A_{648}$ ---- (3.7)

Chlorophyll *b* (mg/g F.W.) = $25.48*A_{665} - 7.36*A_{648}$ ---- (3.8)

Chlorophyll total (mg/g F.W.) = $7.49*A_{665} + 20.34*A_{648}$ ---- (3.9)

where, A_{665} = absorbance at 665 nm, A_{648} = absorbance at 648 nm, F.W. = Fresh weight.

3.3.16 Preparation and modification of peanut shell biochar

PSB was prepared by using PS as feedstock as described by Chu et al. (2017). PS feedstock was pyrolyzed at a rate of 10^0C/min for 2 h with different temperatures maintained at 300ºC, 400ºC, 500ºC, 600ºC and 700ºC, respectively. Pyrolysis conditions play an important role in development of biochar with desirable properties (Katiyar et al., 2021). After the preparation of PSB, most efficient biochar (obtained after initial experiments for % removal efficiency) was selected for modification (MPSB). The modification was carried out by using the procedure as described by An et al. (2019).

In brief, 5 g of PSB was mixed with 10 mL of 0.5% $KMnO_4$. The mixture was stirred for 4 h at room temperature and oven dried at 80ºC. Later, 15 g KOH was mixed with the oven-dried biochar and it was then heated at 300ºC for 1 h. The obtained biochar was repeatedly washed until the spent water had a pH of 7. The biochar was then dried at 60ºC till a constant weight was obtained. Figure 3.6 represents the procedure involved in the preparation and modification of PSB. A duration of 2 h was selected for the preparation of biochar because an increase in duration at low temperatures results in a reduction in biochar yield, whereas an increase in duration at high temperatures has little effect on biochar yield and pH (Sun et al., 2016).

3.3.17 Effects of parameters on arsenic removal efficiency of the biochar

The effect of various parameters such as pH, adsorbent dose, contact time, initial concentration, temperature and rotation speed were studied for optimization of the adsorption process for removal of arsenic. pH was varied from 2 to 11 using HCl and NaOH, adsorbent dose was changed from 0.3 g/L to 3 g/L. Contact time provided was varied from 5 min to 720 min, initial arsenic concentration for both, As(III) and As(V),

ranged between 50 µg/L to 1000 µg/L. Shaking (rotation) speed was varied from 60 rpm to 120 rpm and temperature between 25°C to 45°C. One parameter was optimized at a time and the optimized value was then fixed for further experiments while other parameters were varied.

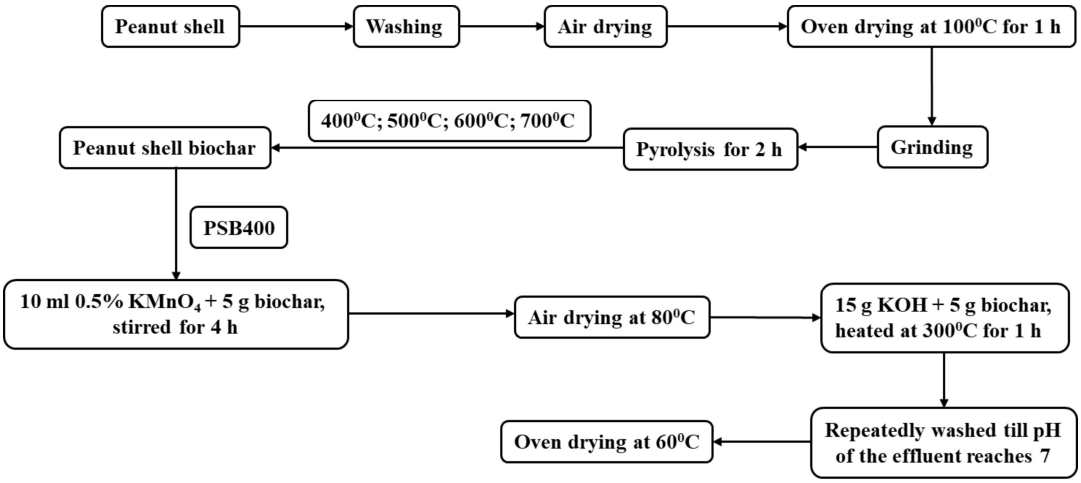

Figure 3.6: Schematic diagram of procedure for preparation and modification of PSB (PSB400 represents the PSB prepared at 400^{0}C used for further modification)

3.3.18 Adsorption isotherms and kinetic studies

Langmuir and Freundlich isotherm models were used to describe the adsorption isotherm process and pseudo-first order, pseudo-second order, Elovich and intra-particle diffusion models were used for the kinetic modeling. The equations for Langmuir and Freundlich isotherms have been described below (Shan et al., 2020):

Langmuir isotherm: $\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{q_m K_l}$ ---- (3.10)

Freundlich isotherm: $q_e = K_f c_e^n$ ---- (3.11)

where, q_m = maximum adsorption capacity (mg/g), C_e = equilibrium ion concentration (mg/L), q_e = equilibrium adsorption capacity (mg/g), K_f = Freundlich constant, n = heterogeneity factor, K_l = Langmuir isotherm constant.

The equations used for kinetic modeling are (Kirbiyik et al., 2016):

Pseudo-first order: $\ln(q_e - q_t) = \ln q_e - k_1 t$ ---- (3.12)

Pseudo-second order: $\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$ ---- (3.13)

Elovich model: $q_t = \frac{1}{\beta}\ln(\alpha\beta) + \frac{1}{\beta}\ln(t)$ ---- (3.14)

Intra-particle diffusion model: $q_t = K_p\sqrt{t} + C$ ---- (3.15)

where, q_e = equilibrium adsorption capacity (mg/g); q_t = quantity of adsorbate adsorbed (mg/g) at time t (min), k_1 and k_2 = rate constants, α = initial adsorption rate (mg/g-h), β = desorption constant (g/mg), K_p = intra-particle diffusion rate constant (mg/g-$h^{1/2}$), C = intercept (mg/g).

3.3.19 Immobilization of bacteria on biochar and preparation of beads

Immobilization of bacteria on biochar was carried out as per the steps described by Tu et al. (2020). TY6 and K7Pb were separately inoculated in LB media at 120 rpm and 30°C for 8 h. Five grams of pre-autoclaved PSB was added to the culture in a 1:20 (w/v) ratio and incubated for 24 h before application. The bacteria immobilized on biochar (IB) was then used to prepare the beads. Beads were prepared using PVA, SA and $CaCl_2$. Proportions of PVA, SA and $CaCl_2$ were varied in order to optimize their concentrations. PVA (1%, 3%, 5%, 7%, 9%) and SA (0.5%, 1%, 1.5%, 2%, 2.5%) were heated at 100°C until the chemicals were completely dissolved in distilled water and were later cooled to room temperature. Then, IB was added to the solution and thoroughly mixed for about 15 min. After that, the mixture was added to the $CaCl_2$ (0.5%, 1%, 1.5%, 2%) solution using a syringe for proper shape formation of beads. They were kept overnight at 4°C to improve cross-linking (Jaiswal et al., 2023b). Beads were washed thoroughly to remove residual calcium chloride from the surface before use. The beads prepared were IBK and IBT, where IBK corresponds to beads of biochar immobilized with strain K7Pb and IBT corresponds to beads of biochar immobilized with strain TY6. Beads of K7Pb and TY6 strains (i.e., consortium) were referred to as IBK+IBT.

3.3.20 Effect of parameters on arsenic removal efficiency of beads

Effects of temperature, pH and initial concentration were studied on the removal efficiency of arsenic. Temperature was varied from 20°C to 45°C (20, 25, 30, 35, 40, 45°C). pH was maintained using HCl or NaOH and was varied from 3 to 11 (3, 4, 5, 6, 7, 8, 9, 10, 11). To study the effect of initial concentration, As(III) and As(V) concentrations were varied from 0.5 mg/L to 100 mg/L (0.5, 1, 5, 10, 20, 50, 100 mg/L).

3.3.21 Water absorption and mechanical strength of beads

A fixed number of beads were immersed in distilled water and were kept for ten days under controlled conditions. The weight of the beads was measured daily to determine the water absorption as per equation 3.16:

$$\% \text{ absorption} = \frac{W_f - W_i}{W_i} \qquad \text{---- (3.16)}$$

where, W_i = initial weight and W_f = final weight at the end of each day.

The mechanical strength of the beads was assessed as described by Tuyen et al. (2018). In brief, 20 beads of similar size were put in a beaker with 80 mL of distilled water. The mixture was magnetically stirred at 500 rpm for 48 h. The ratio of intact beads to the total number of beads was determined.

3.3.22 Arsenic removal in a recirculating packed bed bioreactor using beads

The reactor setup for continuous study using beads was similar to that used in Section 3.3.9, 'Recirculating packed bed bioreactor'. The setup consisted of borosilicate reactor, feed tank, peristaltic pump, sparger, air blower. The working volume of reactor was approximately 1.2 L. The column was filled with 200 – 250 g of beads and influent was fed through a pump. The test was conducted for five cycles and a single cycle was assumed to be completed when the arsenic removal efficiency reduced to less than 70%. Samples were collected daily for analysis of arsenic concentration. The regeneration of beads was performed by washing them using distilled water and agitation was achieved by using a sparger. The flow rate for the entire experiment was maintained at 5 mL/min. A schematic diagram of the reactor setup is shown in Figure 3.7.

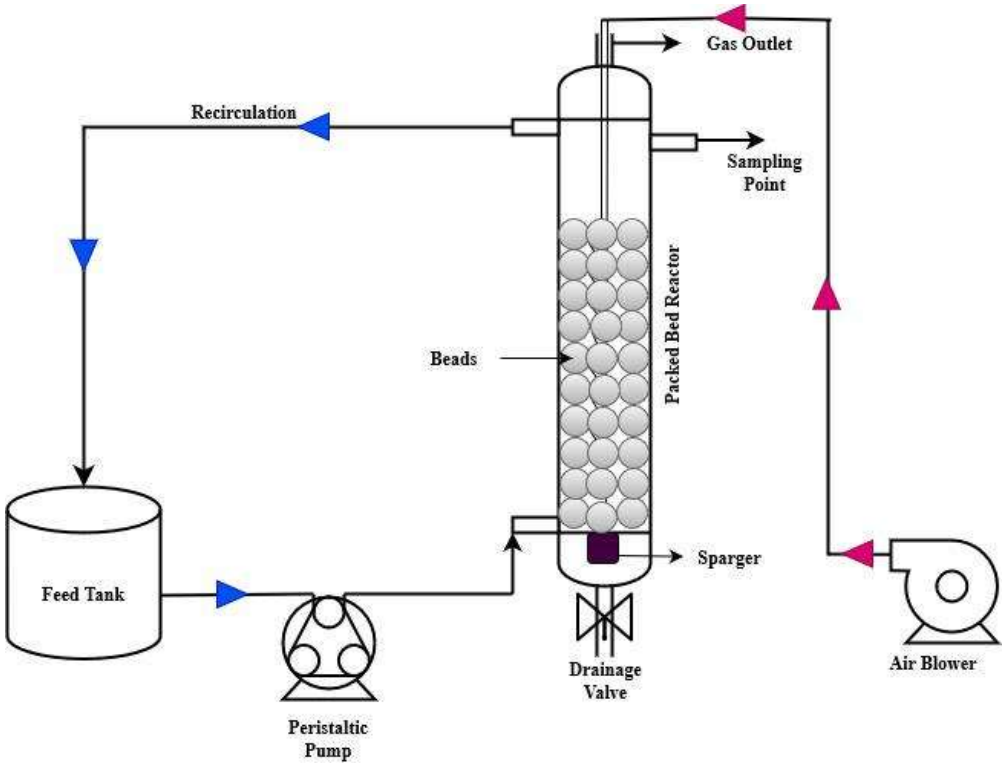

Figure 3.7: Schematic diagram of RPBBR for column study employing beads