Chapter 2
Material
And
Method

2. Materials and Methods

In the present work benzene bio-remediation was studied in immobilized (PBBR) as well as free-cell bioreactor and thereafter investigated in the Continuous - PBBR (C-PBBR) using three bacterial species isolated from petroleum contaminated site at lab scale. The characterizations of bacterial isolates were done by DNA techniques. The process parameters were optimized in the batch mode to obtain the better biodegradation rate. The performance of bioreactors was evaluated under varying process conditions. The kinetic study, identification of metabolites and predication possible bio-degradation pathway, and analysis of identified proteins by bio-informatics servers and tools were also carried out.

This section present details of the materials used for experimental work including bioreactor set-up, methods used for analysis and interpretation of the experimental results, various analytical and other instruments used for characterization and routine measurement work. This chapter and different sections/sub-sections cover different materials used e.g. benzene, other chemicals and reagents, nutrient solution, packing media, bioreactor setup, different methods used e.g. experimental procedure, kinetic study and evaluation of kinetic constants, proteomics analysis, and analysis techniques such as Gas chromatography–mass spectrometry (GC-MS), scanning electron microscope (SEM) ,MS/MS , Matrix-Assisted Laser Desorption Ionization time of flight (MALDI-TOF), Fourier-transform infrared spectroscopy (FTIR).

2.1 Materials

2.1.1Chemicals and Reagents

In the present work, benzene was used as target pollutant for bio-remediation studies because it is one of the major organic pollutants present in the atmosphere due to its large scale

use as solvent as well as raw material for production of other compounds. Analytical grade (>99.9% purity) benzene and other chemicals were procured from Merck India and used in the present study. The chemicals used in experiments are shown in **Table2** with their physical and chemical properties

Table 2: Physico-chemical properties of benzene (Source; Perry, 2000, ASTDR)

Property	**Benzene**
Formula	C_6H_6
Molecular weight	78.11 g/mol
Form and color	Clear colorless liquid
Specific gravity (25°C)	0.876
Vapor pressure (mmHg)	95.19
Melting point	5.50°C
Boiling point (°C) at 760 mm Hg)	80.10°C
Water solubility (mg/l)	1785.5
Density (g/ml)	0.87
Henry's law constant	0.557 atm-m^3/mole
Odor	Sweet smell

In this study other chemicals and reagents are used listed below (**Table 3**). All the chemicals are of laboratory and reagent grade.

Table 3 Chemicals and reagents used in experiments

Reagents	Manufacturers
Nutrient broth: Agarose, yeast extract, tryptone Peptone, Beef extract, K_2HPO_4, H_2PO4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$,$FeSO_4$, $CaCl_2.2H_2O$, Trace elements $CuCl_2.2H_2O$, $CoCl_2.6H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$,$Na_2MoO_4.2H_2O$, and $NiCl_2.6H_2O$	Merk India, Varanasi
EDTA, ethidium bromide, sodium dodecyle sulphate, isopropanol, sodium acetate, lysozyme buffer	
Phenol, chloroform, ethanol, $NaNO_3$	
PVA, Sodium Alginate,	

2.1.2 Materials and Equipments

To carry out the experimental studies various equipment's of standard make such as Refrigerated Incubator shaker, Hot plate, Rotameter, Autoclave, sterilized polybag, parafilm, Micro-pipette and micro-pipette tube, append-off, silicon tube, Sonicator, sprit lamp, Laminar flow apparatus, inoculation loop and peristaltic pumps were used.

2.1.3 Glass wares

The glass wares of standard make such as Shake flasks beakers, measuring cylinders, glass bottles, test tubes, volumetric flasks, petri-dishes, culture tubes, L-shape glass rod, conical flasks, flat bottom flasks, desiccators, round bottom flasks, serum and reagent , bottles etc. were used which were made up of boro-silicate glass were used for the experimental and analytical studies. Prior to use all the glass wares sterilized by sulphuric acid and potassium dichromate and thereafter rinsed properly with distilled water.

2.1.4 Packing material: Poly Urethane foam and alginate beads

Role of packing media is very important in the immobilization and consequently biodegradation of the pollutants in the bioreactors. In the present study Polyurethane Foam and Alginate Beads were selected as packing material due to their favorable properties as support media.

Polyurethane foam of density 45kg/m^3 and 1cm thickness was bought from the native market of Varanasi, India and were cut into cubes of 1cm^3 each. Moisture retention capacity, porosity, bulk density and solid density were experimentally determined the results came out to be 23.6 kg/m^3, 90%, , 6.23 g/g and 236 kg/m^3respectively (Singh et al., 2010). The polyurethane foam cubes were soaked in 80% (v/v) ethyl alcohol and afterwards cleaned properly with distilled water as shown in Fig 7.

The preparation of packing media PVA-alginate beads were done by mixing 50 mL of 10% (w/v) PVA solution, 50 mL of bacterial culture and 2% (w/v) sodium alginate. The mixture was then heated upto 40 ^{o}C. After that drop-wise addition of saturated solution of boric acid with 2% $CaCl_2$ by a peristaltic pump was done which led to the formation of bacterial encapsulated calcium alginate beads. For providing stability and strength to the beads the mixture was kept for 5 hr at room temperature. Finally encapsulated beads were washed with de-ionized water to remove untrapped bacterial species. For further studies, it was dried, weighed and stored in phosphate buffer (50 mM, pH 7.0) as shown in Fig.8.

Fig.7 Polyurethane foam

Fig.8 PVA-calcium alginate beads

2.1.5 Packed bed bioreactor (PBBR)

Packed Bed Bio-reactor of cylindrical borosilicate glass (height = 66cm; I.D(inner diameter) = 6cm) of total volume 1866 mL(milliliter) and working volume 1200 mL was designed for benzene degradation as depicted in Fig.9. The top and bottom outlet ports were provided at a height of 44cm and 4cm respectively for sample collection. The air supply through sparger was from the bottom of the reactor at 1 L/min by an air compressor through 0.20-µm membrane filter to maintain aerobic condition in Packed Bio-reactor. The spargers have four radial arms at 90^{o} apart for uniform circulation of air from the bottom to top in the bio-reactor. The reactor was packed upto a height of 44 cm with Alginate beads with immobilized bacteria and PUF. Benzene with concentration of 400 mg/L was fed into the bio-reactor packed with immobilized Alginate beads and PUF. All the experimental

were conducted triplicate. An experiment with control benzene concentration was done to understand the change w. r. t time due to transfer of benzene to the air stream and adsorption but the outcomes were insignificant.

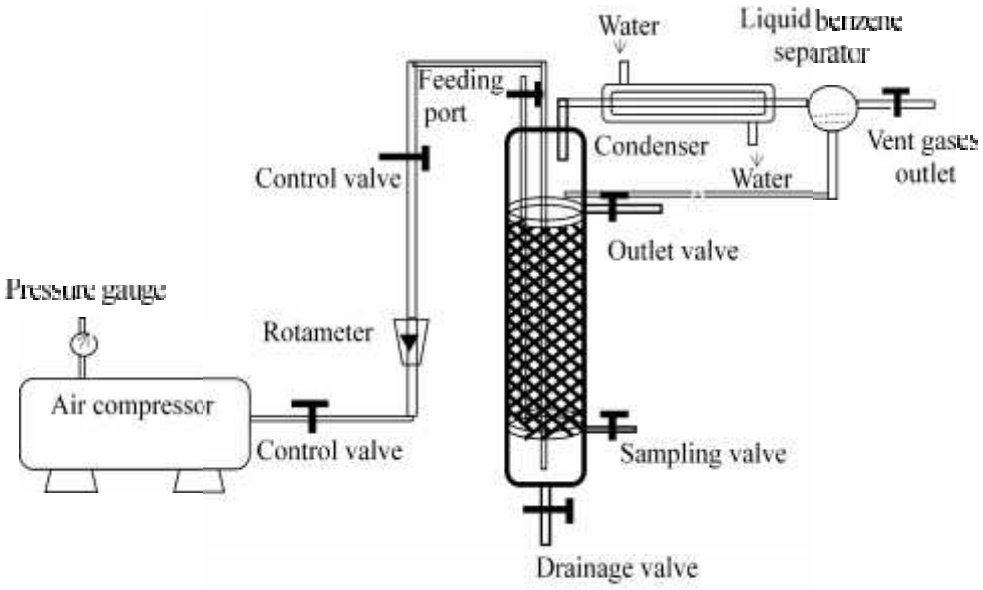

Fig 9: Schematics of experimental setup of PBBR reactor

2.2 Methods

2.2.1 Site description and soil collection:

The soil samples were taken from a transformer oil contaminated site near DLW Hydroelectricity Plant, Bhikharipur, Varanasi, India [129m above the mean sea level and $25^{o}26^{'}$ N, $82^{o}92^{'}$E]. The logic behind the site selection was that the site was contaminated by various organic compounds including ring compounds (constituents of transformer oil) from long time. Fig.10 depicts the collected soil-sample in sterilized polybag.

Fig10: Collected soil sample

2.2.2 Isolation and enrichment:

The bacterial species which can utilize benzene were isolated from the soil using mineral salts medium (MSM).The enrichment of bacterial species was done in a MSM media containing (g/L) of KH_2PO_4 (3.48); K_2HPO4 (4.27); $MgSO_4.7H_2O$ (0.46);$(NH_4)_2SO_4$ (0.34); $CaCl_2.2H_2O$ (0.018); $FeSO_4$ (0.001);. Trace elements $CoCl_2.6H_2O$ (0.2); $ZnSO_4.7H_2O$ (0.1); $Na_2MoO_4.2H_2O$ (0.03); $MnCl_2.4H_2O$ (0.03); $NiCl_2.6H_2O$, (0.02) ; and $CuCl_2.2H_2O$ (0.01); and in mg/L were also added in the media (Tsai et al 2013)**.** The pH was kept at 7 ± 0.1. For enrichment, 5gram of soil sample was inoculated into 100 mL Erlenmeyer flask that contained benzene concentration varying from 50 to 1000 and 50 ml MSM, afterwards the complete setup was placed in an incubator shaker which was maintained that best temp 37 °C for 1 week. The maximum growth was noticed in a flask inoculated with 250 mg/L of benzene. This suspension was further serially diluted from 10^{-1} to 10^{-9}. From each dilution (10^{-1} to 10^{-9}), 100 µL of the suspension was inoculated with 100 µL of culture and afterwards transferred to nutrient broth-agar plates (Fig.11). The microbial colonies were noticed on the plates and thereafter transferred into MSM agar plate where benzene was the sole carbon source as depicted in (Fig.12**)**. Pure cultures of

bacterial isolates were obtained by repeated plating over benzene with MSM agar medium. The growth of cell was determined by measuring optical density of sample against control at 600 nm by spectrophotometer (Elico SL 210, India). Control was simply MSM with no benzene. The two isolates retrieved by above method were stored for further characterization and biodegradation experiments (Kureel et al 2016)

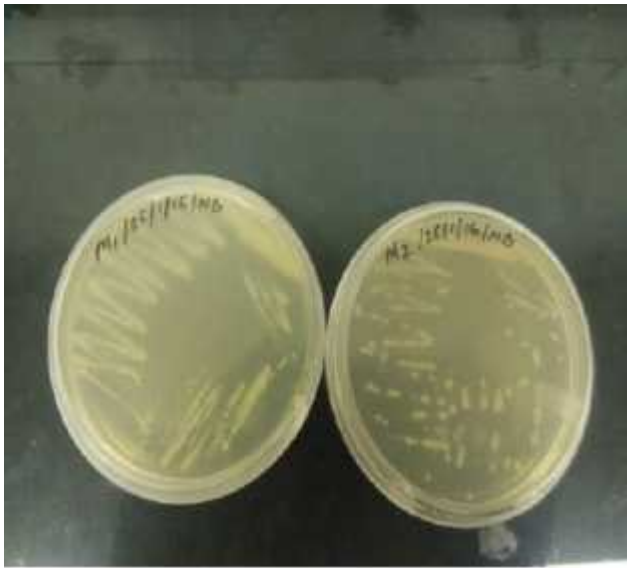

Fig.11: Cell growth in presense of nutrient broth agar on petrides plate

Fig.12: Cell growth in presense of MSM agar media and benzene on petrides plate

2.2.3 Molecular characterization of bacterium isolate

Bacterium genomic DNA was extracted using the standard protocols of Sambrook et al., (2001). Details are described in elsewhere (Shukla et al., 2010).Genomic DNA was subjected to PCR amplification of 16S rRNA gene with universal primers Bac8F (5′AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T3′) (Edwards et al., 1989 and Stackebrandt et al., 1993).The DNA amplification was carried out in a Thermocycler (Bio-rad Laboratories, Inc, Australia). PCR reaction mixture was prepared in a final volume of 50 µL containing 10 mM tris-HCl, 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, 1 IU of Taq DNA polymerase, each primer at a concentration of 0.2 mM, and 1 µL of the DNA template. Each cycle consisted of initial denaturation temperature at 94°C for 2 minutes followed by 30 cycles of amplification program comprising of a denaturation step at 94°C for 50 seconds, annealing at 48°C for 30 second and extension at 72°C for 1.3 minutes and final extension was 72^{o}C for 6 minutes. 5 µL of the amplified mixture was then analyzed using 1% agarose gel and electrophoresis. The gel was stained with ethidium bromide and visualized under UV light, and photographed on gel documentation system.

PCR products were purified before sequencing using PCR purification kit (Axygen, USA) and then sequenced by automated DNA sequencer 3100 DNA Analyzer from Applied Bio-systems using Big Dye Terminator v3.1 cycle sequencing Kit (Applied Biosystems, USA). The sequences obtained were first determined the percentage of similar nucleotides to 16SrRNA gene sequences in the Gen Bank using BLAST program available in NCBI (National Centre for Biotechnology Information) database. Further, the sequences were aligned manually with published sequences in NCBI database using CLUSTALW multiple sequence alignment programs. The evolutionary distances were computed using the

Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The Neighbour-joining algorithm (Saitou & Nei, 1987) was used to generate the phylogenetic tree. Evolutionary analyses were conducted in MEGA6.The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.02682384 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1500 replicates) are shown next to the branches (Tamura et al., 2011). 16S rRNA gene sequence data of bacterial isolates have been submitted to the Gen Bank database under accession number **KU845307-KU845308**

2.2.4 Biochemical test for bacterial isolates

The biochemical characterization of the bacterial isolates were done as per Bergy's Manual of Determinative Bacteriology standard protocol (Holt et al., 1994) Biochemical test kit was used to check the biochemical activity of bacterial isolate as shown below in (Fig.13**)**.

Fig.13 The biochemical kit for idendification of bacterial isollate

2.2.5 Batch study

2.2.5.1 Optimization of process parameters, bacterial growth and biodegradation of benzene in free cell

Firstly in order to increase the cell-density for bio-remediation studies, bacterial isolates were pre-cultured in nutrient broth. Bacterial inoculum was inoculated in 100 mL MSM in separate serum bottles containing benzene. These serum bottles were sealed with aluminum crimp capsbutyl rubber stoppers. Control serum bottle without bacterium inoculum was used. The bacterial development was checked by measuring the absorbance (SL-159, Elico UV-VIS, India) at 600 nm.

In order to select the best isolated species for benzene degradation, batch experiments were conducted. The process parameters were first optimized by varying the process conditions temperature (25.0-43.0 ^{o}C), inoculum size (2.0×10^{8} to 6.0×10^{8} CFU/mL) and pH (5.0-9.0) in the benzene concentration range of 100-400 mg/L. Further experiments were processed on optimized values and potential bacterial species showing good degradation were selected for PBBR and C-PBBR experiments. All the experiments were operated for ten days and triplicate to minimize any error. The average degradation rate of benzene was computed at optimal temperature inoculum size and pH at various concentration using the following equation:

$$r_{ave}(d^{-1}) = \frac{Co-Ct}{CO(\Delta t)} \qquad (I)$$

Where, C_o is initial concentration at t=0 ,C_t is concentration at any time=t , $r_{ave}$is degradation rate and Δt is the time taken for degradation. Biodegradation was checked by GC analysis perform everyday and observed the concentration of outlet decreased continuously and formation of metabolites were found by GC-MS analysis.

2.2.6 Bioreactor study

The reactor was packed upto a height of 44 cm with immobilized alginate beads and PUF and operated at benzene concentration of 400 mg/L. In order to minimize various kinds of errors, all the experiments were conducted in triplicate. An experiment with control benzene concentration was performed to understand the change w. r. t time due to adsorption or transfer to the air stream but the outcomes were insignificant.

3.2.7 Biodegradation kinetics

Several batch experiments were conducted with benzene concentration varying from 100 to 1500 mg/L to study the substrate inhibition in the process. The microbial growth kinetics and substrate utilization was described by Monod model (Eq. II) in non-inhibitory condiation and kinetic parameters were calculated.

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

In the above equation, μ_{max} is maximum specific growth rate (1/hr), μ is specific growth rate (1/hr) and K_S is half saturation rate constant (mg/L). S, t and X denotes initial substrate concentration, time and microbial cell respectively. Above equation was rearranged and integrated to get Eq. III in which X_t is microbial cell concentration at time t and X_o is initial concentration at time t_o.

$$\mu = \log \frac{\left(\frac{X_t}{X_o}\right)}{(t - t_o)} \qquad \text{(III)}$$

The constant K_m is replaced by K_s because bacterial cell activities are measured by using intact cells rather than purified enzymes (Futamata et al., 2005). The Monod model is only valid in case of non-inhibitory condition (Monod, 1949) so for inhibitory condition others models are required in order to compute the kinetic constants. Haldane-Andrews model is most widely and frequently used under substrate inhibition condition (Haldane,

1930; Andrews, 1968) which consist of Inhibition constant K_i(mg/L) as depicted in Eq. (IV) and also used in the current work.

$$\mu = \mu_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}} \qquad \text{(IV)}$$

2.2.8 Continuous study

2.2.8.1 Optimization of process parameter in batch bioreactor immobilization experiment

For optimizing inoculums size, DO and pH batch experiments were performed at lab scale in PBBR. The inoculum size, DO and pH were changed in the range of 2.0×10^8 to 8.0×10^8 CFU/mL, 3.0 to 7.0 mg/L and 3.0 to 10.0, and at 23±2°C room temperature respectively with fixed benzene concentration of 200 mg/L. DO, pH was determined by DO and pH probes (YSI 5100, USA). In order to optimize the process parameters only one parameter was varied at a time and while keeping other parameters fixed. In the first set of optimization experiments, pH was varied from 3.0 to 10.0 at fixed inoculums size 2.0×10^8 CFU/mL, DO (5.0 mg/L) and benzene concentration of 200 mg/L. In the next set experiments, the above obtained optimal values were kept fixed for further experiments. Under optimal condition a, benzene concentration upto 400 mg/L was used to estimate the behavior of CPBBR. ANOVA was also applied to study for minimization of experimental error. Optimum values of parameters obtained were used in packed reactors (PBBR and CPBBR) to maximize the percent removal of benzene.

2.2.9 Packing material and adsorption studies

Polyurethane foam sheet was bought from the native market (Prakash Stationary, Lanka, BHU, India) and cut into approximately $1cm^3$ size. The pieces were cleaned with distilled water followed by ethyl alcohol. It was then squeezed and dried in an

oven at 60^0C for the whole night to release absorbed water. Thereafter, the pieces were packed in Continuous packed bed bioreactor (C-PBBR). The batch adsorption study of benzene on PUF was performed in serum bottles which was shielded and closed by aluminum crimp and cap respectively at concentration of 200 and 250 mg/L. PUF pieces were placed in both bottles and then rotated by a rotary shaker at 150 rpm for 120 h. Benzene concentration in all bottles were analysed using GC at regular interval of 12h. The outcome indicated that PUF's adsorption capacity was nearly negligible for benzene. A detail procedure has been described by Singh et al 2010 and Geed et al 2017.

2.2.10 Continuous packed bed bioreactor: startup, operation and performance evaluation

Continuous packed bed bioreactor (CPBBR) of cylindrical borosilicate glass (height = 55cm; I.D = 6cm) of total volume = 1554mL and working volume = 1000 mL was designed and fabricated for benzene degradation. The top and bottom outlet ports were placed at 44 cm and 4 cm of height, respectively to collect samples. (**Table.4**). Sample collection ports were closed with silicon tubing and pinch cork. A 0.2-µm filter paper was used within the flow-line of outlet port to prevent bacterial species draining from the bio-reactor. In order to maintain aerobic condition and proper mixing, air from compressor (Khosla, India S.N.65739) was supplied through air sparger to the bio-reactor. The rate of aeration was governed by a calibrated rotameter (Eureka, Pune, SRSMG5) and varied in the range of 5 to 30 L/min range. Recycling of gaseous benzene was done by a condenser as depicted in **(**Fig. 14**)**. The outlet stream was collected into a tank and experimental setup shown in **(**Fig. 15**)**.

The behavior of CPBBR was described in terms of EC, % RE and loading rates as given in Eq. V, VI and VII.

$$\% \text{ Removal Efficiency (RE \%)} = \frac{S_{in} - S_{out}}{S_{in}} \times 100 \quad \text{(V)}$$

$$\text{Elimination Capacity (EC)} = Q\frac{(S_{in} - S_{out})}{V} \quad \text{(VI)}$$

$$\text{Inlet Loading Rate (ILR)} = \frac{S_{in}}{V} Q \quad \text{(VII)}$$

Where, S_{in} (mg/L) and S_{out} (mg/L) are the inlet and outlet benzene concentration. Q(mL/h) is the volumetric flow rate of feed, V(mL) is the working volume of bio-reactor.

Table 4: Details of performance and dimension of bioreactor under optimum operating conditions for biodegradation benzene.

Parameters	**C-PBBR**
Height of the packed bed (cm)	55
Diameter of reactor (cm)	6
Total volume (mL)	1554
Working volume (mL)	1000
Void volume (mL)	932.7
Packing material	PUF
Packing material size (cm^3)	1
pH	7.0±0.2
Temperature (^oC)	32±5
DO (mg/L)	5.2
Inlet concentration (mg/L)	400
Loading rate (mg/L/day)	96-480
EC (mg/L/day)	28.8-156
RE %	>90
Operation time (day)	104

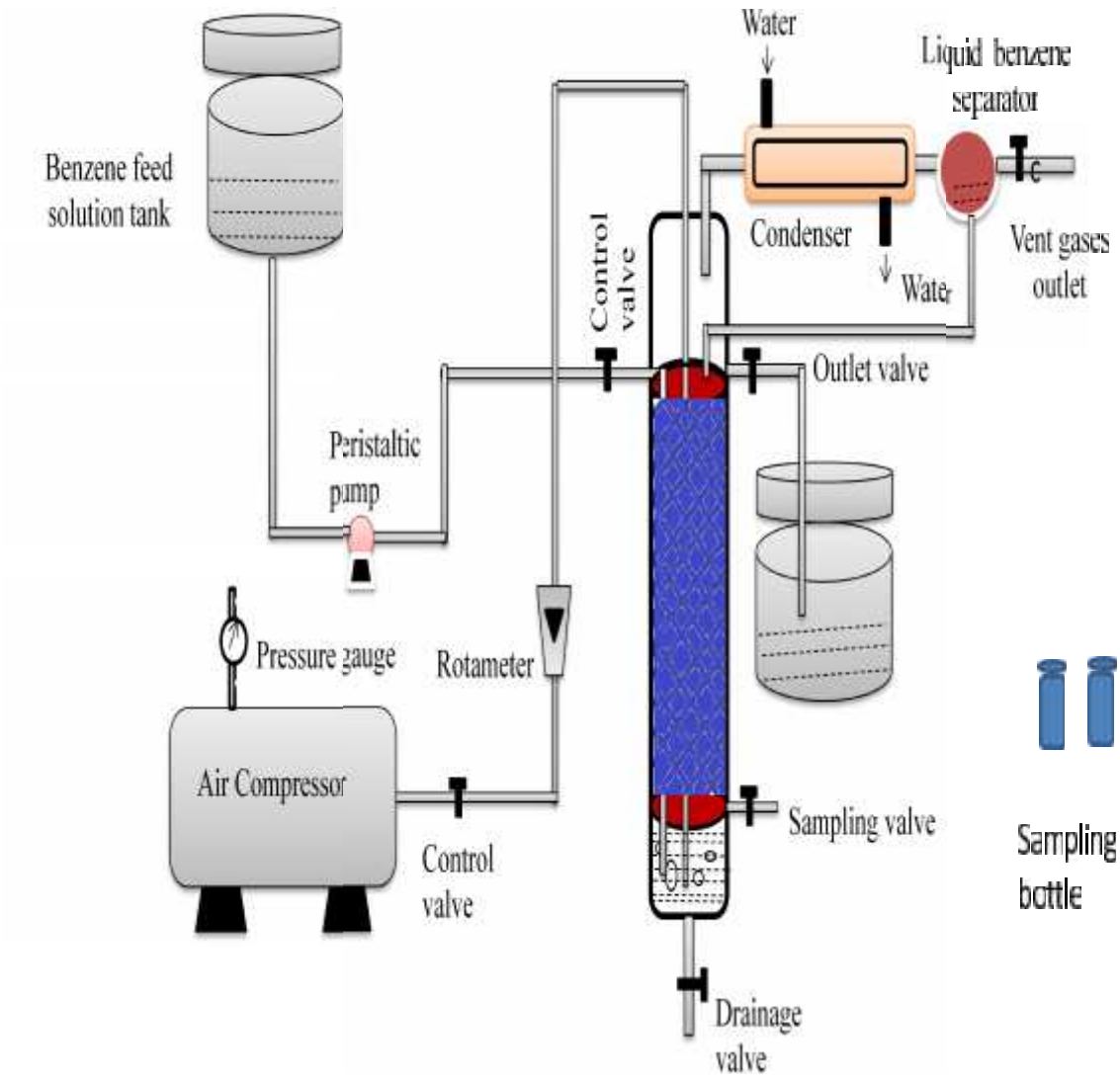

Fig 14: Flow diagram for continuous bioreactor system (CPBBR)

Fig.15 Image of experimental setup of continuous packed bed bioreactor

2.2.11 SEM, FT-IR, GC and GC-MS analysis

Scanning Electron Microscopy (SEM) was done to get morphology of bacterial species.The bacteria from samples were filtered using 0.2 µm cellulose filter paper (Ultipor N_{66} (Nylon 6,6 membrane) manufactured by and Made Pall Life Sciences, India. Thereafter, the filter paper was dried for over-night at 37^0C in an oven and gold coating was done to have better resolution. Finally, the sample was examined using SEM (QUANTA 200F, Netherland) at very low pressure or vacuum to in order to minimize the harm effect on bacterial samples (Robledo-Ortz et al., 2011).

FTIR (NICOLET 5700 FTIR, Japan) analysis of sample taken after 10 days was performed. The spectrum and bands of functional group were recorded in the range of 400 to 4000 (1/cm) (Robledo-Ortz et al., 2011). Benzene concentration was analyzed by (THERMO-FISHER 7610, USA) gas chromatograph (GC) equipped with a BP-5 capillary column (25m×0.32mm, stationary phase consisting 5% Diphenyl – 95% Dimethyl polysiloxane) and Flame-Ionization Detector (FID). In GC operation nitrogen gas was served as a carrier while hydrogen and oxygen gas served as fuel (Liu et al. 2010). Initially the temperature of oven was kept at 50 °C for 30 seconds and then raised at a rate of 10 °C to 80 °C and maintained for 30 seconds. Then it was raised to 150 °C at a rate 10 °C. The detector and injector temperature were kept at 170 ^{0}C and 160 ^{0}C, respectively.

The analysis of Intermediary products or metabolites produced during benzene bio-degradation was done using GC-MS (QP2010 Ultra, Shimadzu, Japan) which was equipped with DB-5 fused silica capillary column (30m×0.25mm×0.25 µm). The column temperature of GC-MS was maintained at 80°C for 2 min and thereafter increased to 250°C at 7°C/min and then to 280°C at 10°C/min**.** The injector temperature was maintained at 260°C (Singh and Fulekar, 2010). For the separation of metabolites, all samples were centrifuged at 8000

rpm for 10 min followed by acidification with hydrochloric acid and finally two times extraction with n-hexane (Kureel et al 2017).

2.2.12. Proteomic study: extraction, quantification and 2-Dgel electrophoresis

Cells of *Bacillus* species treated as well as control were harvested by centrifugation (10,000× g, 10 min.) and suspended in 2 ml extraction buffer containing 10 mM Tris-Cl (pH 8.0), 1.5 mM $MgCl_2$ and 10 mM KCl in the presence of 10 µMPMSF (Sigma), ground in liquid nitrogen followed by centrifugation at12,000× g for 1 h. The supernatant containing cytosolic protein (500 µl) was cleaned using 2DE clean up kit (GE Healthcare Bio-sciences, USA) following the manufacturer's instructions and precipitated proteins were solubilized in a buffer containing 7 M urea, 2 M thio-urea, 4% CHAPS, 40 mM DTT and 1.0% IPG buffer of pH range (3–10) and the protein concentration was measured using the method of Bradford (1976). For IEF, traces of bromophenol blue were added to the protein sample and centrifuged at 19,000× g for 10 min. Then 300 µl of solubilization buffer containing~250 µg protein samples were incubated with dry IPG strips (pH 3–10;13 cm; GE Healthcare, USA) at 20 °C for 12-16 h at room temperature. The focusing was performed at 20 °C with an Ettan IPGphor system (GE Healthcare, USA) in 6 steps: linear 150 V for 2.00 h, 500V for4:00 h, 1000V for 1:00h,8000 V for 2 h, gradient 8000V for 6 h, and finally 8000 V for 8 h. Focused IPG strips were equilibrated by first incubating in an equilibration solution (6 M urea,30% glycerol, 2% SDS, 50 mM Tris–HCl, pH 8.8, and 1% DTT and a trace amount of bromophenol blue) for 15 min followed by incubation in2.5% iodoacetamide in the same equilibration solution of 1% DTT for 15 min. The strip was placed on the top of 12.0% SDS-PAGE and sealed with 0.5% agarose. Electrophoresis was carried out at10 mA/gel for 30 min followed by 25 mA/gel for 5h using a Hoefer SE600

apparatus (Amersham Biosciences, USA). The gels were stained with CBB R-250.2.6. For the analysis of gels, 2DEgelimage analysis software PDQuest™ Basic version 8.0.1(Bio-Rad) was used for gel to gel matching, spot detection, quantification, identification of differences in spot intensities, background subtraction and spot matching between treated and untreated/control for spot quantification (as spot volumes, intensity × mm^2). Missing spot volumes were estimated from the data set employing a sequential K-nearest neighbor algorithm using R 2.7.0 environment. After missing value imputation, total spot volume per gel was used to normalize spot intensities (% of individual spot volume/Σ% spot volume of each gel) to compensate for variations between gel replicates. These data sets were also cubic-root transformed to reduce the spot volume-spot deviation dependency. A total 275 protein spots were identified from gel having statistically significant (p<0.05) and reproducible changes with an abundance ratio of at least 1.5 fold were taken as differentially expressed proteins. The protein spots of interest were manually extract from the gel and processed for MALDI-TOF MS/MS identification (Singh et al., 2015; Rai et al., 2014).

2.2.13 Protein digestion and identification by MALDI-TOF MS/MS

Samples for MALDI-TOF analysis were prepared as per the protocol of Bruker Daltonics (Shevchenko et al., 2006). 100 ml of wash solution (1:1 ratio of acetonitrile and 100 mM ammonium bicarbonate) was added to excised spots and incubated for 30 min for de-staining. It was further incubated in absolute acetonitrile for 3 min and pellets were air dried. Reduction of the protein spots was done using 10 mM DTT dissolved in 100 mM NH_4HCO_3 for1 h at 50 °C. The samples were further alkylated using 55 mM iodoactamide in 100 mM NH_4HCO_3 for 45 min in dark at room temperature. The gel pieces were

dehydrated with 100% acetonitrile, air dried and covered with 20 µl of 25 µg/mL sequencing grade trypsin solution (Promega Gold, USA) and incubated at 37 °C. The polypeptides were eluted from the gel piece in 2.5% trifloro acetic acid (TFA) in 50% acetonitrile by vortexing for 15 min. Extracted peptides were concentrated by vacuum assembly and 1 µL of trypsinized peptide samples was mixed with matrix, CHCA (2.5 mg/ml in 50% acetonitrile) on the target plate(400 well stainless steel, Bruker Daltonics, Germany) and subjected to Auto flex speed MALDI-TOF for mass spectrometric identification.

AB Sciex Protein Pilot software v2.4 with Mascot search engine v2.4 (Szewczyk et al., 2014) was used for protein database searches. The data were searched against the NCBInr (The National Centre for Biotechnology Information).The data for BLAST searches consisted of sets of single spot peptide sequences found with the use Data Explorer software (AB Sciex, USA).

The analysis was conducted on an AB Sciex 5800 TOF/TOF^{TM} system (AB Sciex, USA). Samples were placed on the MALDI plate five times to cover the selection of the 50 strongest precursors for MS/MS analysis. The TOF MS analysis was done in the mass range of 800-4000 Da, laser intensity of 3600 watt/m^2 and pulse rate of 400Hz. The precursor selection order in this mode was set from strongest to weakest. Instrument in TOF MS mode was first externally calibrated and then internally calibrated for every sample with 842.510 m/z and 2211.106 m/z (trypsin autolytic peptides). The TOF/TOF MS/MS analysis was conducted in the mass range10-4000 Da, 4550 V/400 Hz laser relative power, CID gas (air)switched on at a pressure of ca 7×10^{-7} and up to 4000 shots perprecursor with dynamic exit. The precursor selection was set from weakest to strongest

in this mode. The external calibration of MS/MS mode with the fragments of Glu-fibrinopeptide (1570.677 m/z) was applied (Singh et al., 2015; Shrivastava et al., 2015).

2.2.14 In silico analysis and characterization of identified proteins

2.2.14.1 Functional domain analysis, Phylogenic classification, Structural prediction, Active side analysis and Docking

Functional domain analysis of identified proteins was done to predict enzymes family classes and their functional roles. For functional domain classification PROSITE server was used (Sigrit et al., 2012). PROSITE is a protein domain data base for functional characterization and annotation (Sigrits et al., 2012). Phylogenic classification is orderly arrangement of organisms as texa or gene using hierarchical series. Phylogenic classification of depend on characters used in its construction. Sequential classification reveals the close and distant relationship among the selected homologous gene for phylogenetic classification of multiple alignment sequence was performed using clustalw (Thompson et al., 1997). For phylogenetic construction MEGA6 tool was used (Tamura et al., 2013).

Structural prediction was done to characterize the hypothetical and identified proteins using homology modeling (Vyas et al., 2012). The homology modeling refers to constructing an atomic resolution model using experimental three dimensional structure of related homologous protein (template) (Arnold et al., 2006). For prediction of good quality the model sequence identity should be >20% , below 20% sequence identity can have very different structure (Arnold et al., 2006). Quality assessment was performed by RAMPAGE and structural classification was performed using CATH server (Aamir et al., 2017). Further best predicted model was taken BAI84687.1 (Hypothetical protein) for docking

calculation. Molecular docking analysis was performed using YASARA (Yet another Scientific Artificial Reality Application) (Sessa et al., 2016). Active sites and residues were identified using META Pocket server (Huang, 2009). The best predicted model was taken for docking calculation with benzene (CID:) and docking complex was visualized using Discovery Studio 3.0.