3.1 Experimental

The present work is concerned with the degradation of organic pollutants by biodegradation (i.e. using indigenous microbes) and photo-catalytic degradation (i.e. using heterogeneous catalysts). For biodegradation study, various microbes were isolated from the contaminated sites (i.e. water and soil), whereas catalysts were synthesized in laboratory for photochemical degradation. Further, the catalysts were characterized using various techniques such as morphology, surface characterization and thermal stability. Details of the chemicals and materials used and procedures employed are given in the following sections

3.2 Chemicals

Benzene (purity, 99.7%) and toluene (purity, 99%) were used in the degradation process. In addition, concentrated sulphuric acid, potassium dicromate, starch indicator, ferrous ammonium sulphate, mercurous sulphate ($HgSO_4$), potato dextrose agar (PDA), nutrient agar (NA), sodium hydroxide (NaOH), and ethanol are also used in different experimental processes.

3.3 Isolation, screening and identification of petrochemical degrading microbes

3.3.1 Media preparation

Reagents: Nutrient Agar (NA), Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) plates.

For the preparation of NA media 500 ml distilled water was taken in a 1L conical flask. 14 gm of Nutrient agar powder, and 7.5 gm 100 ml^{-1} of agar powder were mixed well in the distilled water and the volume was further maintained to 1000 ml. The solution was heated for a few minutes (5 minutes) in order to get homogenate solution. The media was autoclaved at 121º C, 15psi, for 30 min. For the preparation of PDA media, 500 ml distilled water was taken in a 1L conical flask. 20 gm of Potato Dextrose Agar (PDA) powder, and 7.5 gm 100 ml^{-1} of agar powder were mixed well in the distilled water and the volume was further maintained to 1000ml. The media solution was heated for a few minutes and autoclaved at 121º C, 15psi, for 30 min.

3.3.2 Isolation and purification of microbial culture

In natural habitats, microorganisms usually grow in a complex manner, having mixed population of various species and strains. It poses difficulty for microbiologists in isolation of a single type of microorganism, which is required for study and characterization for a particular microorganism. In the present study, we used serial dilution technique to isolate microorganisms from the mixed culture in sample.

3.3.3 Serial dilution

(A) Ten test tubes were taken with 9 ml distilled water in each. 1 ml of sample was taken from stock solution/sample and added in the first test tube and the whole solution was mixed thoroughly. From the first test tube, one ml sample was added to the next test tube. Subsequently, the same process was repeated till

the last test tube. Micropipette is used for measuring 1 ml solution. The dilution was performed in sterilized conditions in the laminar air flow.

(B) 20 µL of diluted samples using micropipette were taken from selected test tubes. The diluted samples were properly spread on different plates (NA, PDA, and RBC) with sterilized spreader.

(C) The plates of selective media (NA, PDA and RBC) were transferred in the incubators having different temperatures and waited for 12 hrs for observation of bacteria and 24-48 hrs for observation of fungi.

(D) In this method growing colonies of bacteria or fungi on first round of plate were streaks on another plate of same selective media using inoculation loop under sterilized conditions and incubated on same temperature, as done for the previous plate. The same procedure was followed in third round of sub-culturing/plating to get pure colony of the microbial strains.

(E) The isolated microbial cultures should be sub-cultured after a fixed duration of time for their long term viability and activity. It has been suggested that bacterial cultures should be sub-cultured on every 20 days, while fungal cultures should be sub-cultured every 30 days of interval.

(F) For long term preservation of pure culture, the selective media slants and 30% glycerol stock solution should be used. The microbial cultures preserved in glycerol stock solution should be stored in deep freezer at -80^{o}C.*3.3.4 Preparation of Mineral salt medium (MSM)*

Mineral salt medium (MSM) was prepared by mixing of following chemicals all the chemical used are analytical grade and procure from sigma Aldrich , Merck, Himedia , thermo fisher scientific :

For 1000 ml of medium, KH_2PO_4 (0.5 gm), K_2HPO_4 (0.5 gm), $(NH_4)_2SO_4$ (2.0 gm), NaCl (0.01 gm), $MgCl.6H_2O$ (0.2 gm), $CaCl_2$ (0.02 gm), $MnSO_4$ (0.0003 gm), $ZnSO_4$ (0.0004 gm), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0003 gm), $CoCl_2.6H_2O$ (0.0004 gm) and EDTA (0.001 gm) were used, thoroughly mixed and then autoclaved for 30 minutes at 15psi pressure and 121°C temperature.

3.3.5 Analytical investigation

HPLC investigations of toluene and benzene biodegradation were performed as per Rajamanicjam et al. (2016). The detailed processes are briefly described here. Toluene in the liquid phase was analyzed by HPLC (Thermo Scientific Ultimate 3000) chromatograph with UV detector at 254 nm. The column used was C18 bond packed 3 µm (25×4.6 mm) with mobile phase consisting of methanol and water (70:30). The flow rate was set at 1 ml min^{-1} with resistance time of 2.4 minutes. The samples were also analyzed by GC−MS. Inoculated samples were centrifuged for 10 minutes at 10,000 rpm to separate cell mass and the supernatant. Supernatant was extracted in organic solvent (n-Hexane) for analysis through mass spectrometry to identify toluene, benzene and its degraded products. The samples were analyzed by mass spectrometer (Shimadzu Gas Chromatograph attached with single quadruple Mass Spectrometer (GCMS-QP2010 Plus)) using ionization mode, ESI (electron spray ionization) and masrlynx 4.0V software.

3.3.6 Cell growth

The cell growth was determined by measuring absorbance of bacterial culture at 600 nm using UV–Visible spectrophotometer (Systronics, India) (Padhi and Gokhale 2016; Sahoo et al. 2014).

3.3.7 Molecular Identification procedure of selected Bacterial strains

DNA was isolated from the isolated bacterial culture, and its quality was evaluated on 1.2% agarose gel. Fragment of 16S rDNA gene was amplified by PCR using 8F and 1492R primers from the above isolated DNA. The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with **704F** and **907R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of **1412 bp** 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The obtained 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genBank database. Based on maximum identity score, first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

3.4 Synthesis of Activated carbon-TiO_2 composite

3.4.1 Materials

All other chemicals used in this study were of analytical grade. Titanium tetra-isopropoxide (TTIP) and hydrogen peroxide (H_2O_2) were procured from Sigma

Aldrich, India. Toluene (99.5 %), Ethyl benzene, Xylene and Benzene (99.5%) of analytical grade were purchased from Merck, India. Acid Red 131 Dye (CAS No. 12234-99-0) was procured from MILI Industries, Gujarat with empirical formula $C_{30}H_{23}N_3O_9S_2$-H and molecular weight of 645. Direct Blue 199 Dye was brought from Sigma-Aldrich (CAS no. 4399-55-7) with empirical formula $C_{40}H_{28}N_7NaO_{13}S_4$ (Hill notation) and molecular weight 965.94 (approx.). Activated carbon (<20 μm) used for the preparation of TiO_2/Activated-C (TiO_2/AC) nano-composite was purchased from Rankem chemicals, India. Activated carbon was also synthesized from rice husk obtained from a nearby rice mill, by carbonization at 400^oC followed by chemical activation using H_3PO_4 as an activating agent and used in the synthesis of composite.

3.4.2 Preparation of TiO_2/AC- nano-composite

The synthetic procedure for the formation of nano-composite involves sol-gel technique. TiO_2 adsorbed on the surface of AC was used as photo-catalyst for the degradation process. Titanium tetra-isopropoxide (TTIP) was used as binder. Description of the whole process is as follows (Inoue et al. 1994; Horie et al. 1998; Masaki et al. 2007)35.8 gm of TTIP was taken and dissolved in 180 ml of 98% propanol and 20 ml of 34% HCl (w/v), and the obtained mixture was homogenized by keeping it in shaker for an hour. The resulting solution was diluted to 1L ml by manipulating the pH to 3 by addition of NaOH. Further 10 gm of activated carbon and 10 gm of P25 TiO_2 particles were mixed in the resultant solution and stirred for 3 hours. The obtained Gel solution was membrane filtered and oven dried at 85 °C for

24 hours. The dried sample was crushed and calcinated at 350^{o}C The obtained TiO_2/AC was utilized further in the study.

3.4.3 X-ray diffraction (XRD)

BRUKER D8 advanced wide-angle X-ray diffractometer with a graphite monochromator was used for performing X-ray diffraction using Cu-Kα source with a wavelength of 0.154 nm. The generator was operated at 40 kV and 20 mA. The powder or thin sheet of samples, nanofibres and foam were placed on silica sample holder at room temperature (~25 °C) and were scanned at diffraction angle 2θ from 10° to 80° at the scanning rate of 2° min^{-1} (Singh et al. 2015). The peak width at half maxima in the XRD peaks was used to determine the crystallite size as per Scherrer formula:

$$D = \frac{K\lambda}{\beta(Cos\theta)} \qquad (3.1)$$

Where, K=0.9 (X-ray wavelength), λ = 0.154 nm, β is full width at half maximum and θ is the Bragg's diffraction angle.

3.4.4 Brunauer–Emmett–Teller (BET) surface area

BET surface area and pore dimension of the samples were analyzed by nitrogen adsorption–desorption isotherm measurements at 77 °K on an adsorption apparatus (ASAP 2020, Micromeritics Instruments, USA). Prior to measurements, the powder samples were degassed at 180 °C for 6 hours and composite samples were degassed at room temperature for 24 hours. The BET specific surface area was

determined by the multipoint BET method using the adsorption data in the relative pressure (P/P_o) range of 0.01–0.99 and 0.05–0.30 for particles (Singh et al. 2015).

3.4.5 Raman spectroscopy

Raman spectroscopy is one of the spectroscopic techniques based on the polarizability of the molecules present in an organic or inorganic compound under the electric field (laser beam). The spectrograms were obtained at room temperature in backscattering geometry using an Ar^+ excitation source, having a wavelength of 488 nm coupled with a Labram-HR800 with a resolution of 4 cm^{-1} (Singh et al. 2015).

3.4.6 Fourier transform infrared (FTIR) spectroscopy

Infra-red Spectroscopy is one of the most common technique used to determine functional groups in organic compounds. Each functional group absorbs a characteristic frequency of IR radiation. The advantage of FTIR spectroscopy over a conventional dispersive IR Spectroscopy is that this offers a faster and more sensitive analysis. The FTIR measurements were performed in transmittance mode at room temperature from 400 to 4000 cm^{-1} using Thermo Nicolet 5700, USA with a resolution of 4 cm^{-1}. Nanoparticles spectra were recorded by making pellets with 10% particles in KBr powder, and the direct spectra was taken for samples.

3.4.7 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS)

The morphology of the composite was examined using a high resolution Scanning Electron Microscope (SPURA 40, Zeiss) operated at an accelerating voltage

of 2-5 kV. Prior to imaging by using SEM, samples were sputter coated for 50 sec with gold using a JEOL JFC-1200 fine coater to make specimens conductive. Based on the SEM micrographs, particles diameter were analysed using an image visualization software Image-J developed by the Upper Austria University of Applied Sciences.

2.4.8 Photocatalytic degradation experiment

The degradation studies were performed in photo-catalytic reactor as shown in figure 3.1. Dye samples (ranged from 25-200 ppm) were utilized for degradation studies with constant loading of catalysts TiO_2/AC (0.5 g L^{-1}). The dye sample with catalysts was initially stirred for 30 minutes in absence of any light to investigate its adsorption capacity. Thereafter, this sample was placed in cylindrical jacketed quartz holder of reactor. Water at ambient condition was used as coolants. Sample were withdrawn, centrifuged and tested for extent of degradation interval of five minutes and characterized through UV visible spectrophotometer. Photo-catalytic activity of nano-composite over dye at its various concentrations was determined by processing degradation experiments and sample analysis using UV-Visible double beam spectrophotometer. Photochemical reactor was used to perform the degradation. To begin with, 0.05g TiO_2/AC nano-composite was added to each 100 ml dye solution of varying concentrations, and stirred and maintained in dark conditions for 30 minutes to obtain the equilibrium adsorption stage. Thereafter, each (dye + catalyst) suspension was irradiated with UV (under constant stirring) by using Perkit India's Photochemical reactor (λ=185), with overall supply of 64 W UV radiation. During

irradiation period, small amount of suspension was withdrawn at regular interval and centrifuged to separate the catalyst and remaining solution was examined at $\lambda = 570.2$ nm with the aforementioned spectrophotometer. Amount of dye degraded (%Deg.) was assayed using formula as follows:

$$\%\text{Deg.} = \frac{(\text{Co} - \text{C})}{C} \text{x} 100 \qquad (3.2)$$

Where Co = Initial Concentration of dye (before degradation)

C= Concentration of dye after degradation

3.4.9 Design of Reactors

Three reactors were used during the experiments. A photochemical reactor which consisted of a cylindrical reactor fitted in with 8 Phillips UV lamps of intensity 960 LUX along with a magnetic stirrer shows schematic view of the photochemical reactor. For carrying out sonication reaction, a reactor fitted with a Sonicator (Hielsher Ultrasound Technology) was used. Figure 3.1 shows a schematic representation of a reactor consisting of a combined photo- and Sono-catalytic reaction. All these reactors were provided with cooling jackets with water flowing inside them so as to avoid overheating.

Various solutions consisting of different concentrations of dye, along TiO_2/AC catalyst was used for the experiments. The experiments were conducted in the three batch reactors as shown earlier. The samples were taken out from the batch reactor at

fixed time interval and their concentration was measured. This data was then used for further deductions and calculations.

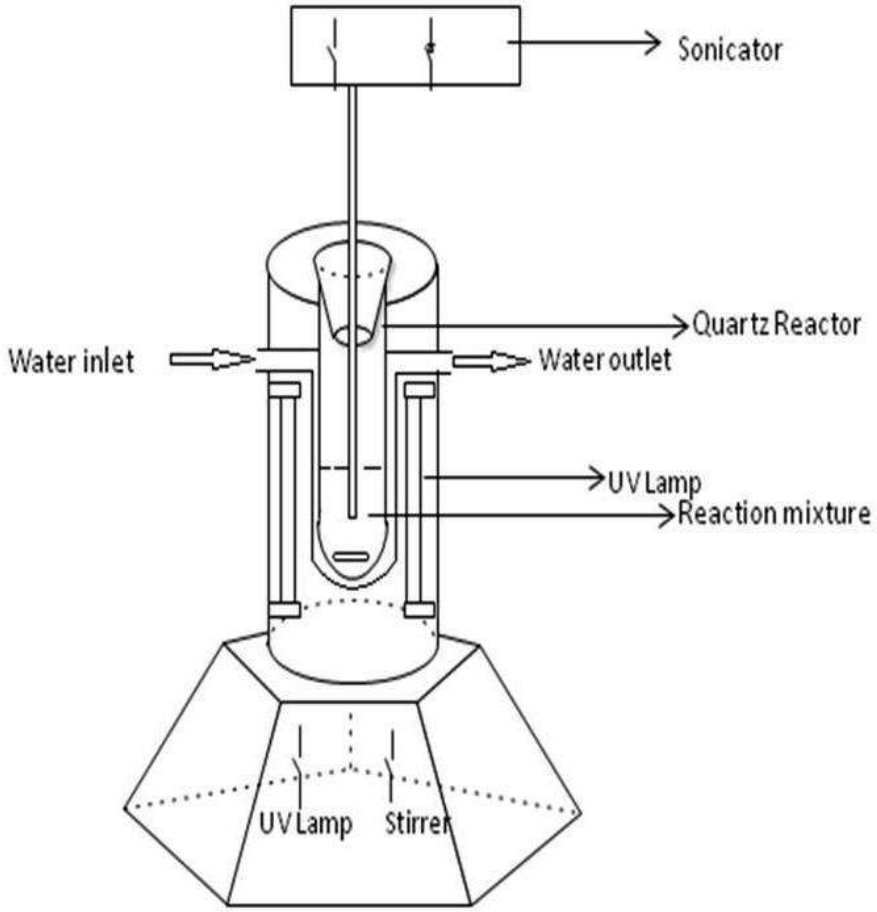

Figure: 3.1 Schematic diagram of photochemical reactor

3.4.10 Preparation of composite suspensions

The TiO_2/AC nano-composites were suspended directly in deionized water and dispersed by ultrasonic vibration for 30 min. Small magnetic bars were placed in the suspensions for stirring before use to avoid aggregation of the particles.

3.5 Seeds

Seeds of important agricultural crops (viz., *Vigna radiata* and *Solanum lycopersicon*) were collected from the Institute of Agricultural Science, Banaras Hindu University, Varanasi, India.

3.5.1 Seed germination and exposure

The collected seeds were washed in tap water initially, then immersed in a 10% sodium hypochlorite solution for 10 min and further rinsed three times with deionized water to ensure surface sterility. These seeds were soaked in nano-composite suspensions solution for about 12 hrs. One piece of filter paper was put into each 100 mm petri-dish, and 5 ml of a test medium was added. Seeds were then transferred onto the filter paper, with 10 seeds per dish with about 1 cm distance between seeds (Yang and Watts 2005). Petri-dishes were covered and sealed with paraffin, and placed in the dark in a growth chamber at 25 ^{o}C. After each 24 hrs, the germination of seeds was checked, on regular basis. The root and shoot length were measured after 3 days when germination was halted, and seedling root length was measured by a millimetre ruler to observe the effects of incubation on the root and shoot elongation.

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