3 Materials and methods

3.1 Materials and Chemicals

Analytical grade Atrazine, Malathion and Parathion procured from Sigma-Aldrich, India with more than 99.0 % purity were used in the experiments. The mineral salt medium (MSM) with composition in mg/L (KH_2PO_4:840; K_2HPO_4:750; $(NH_4)_2HPO_4$:474; NaCl:60; $CaCl_2$: 60; $MgSO_4 \cdot 7H_2O$:60; $Fe(NH_4)_2SO_4 \cdot 6H_2O$:20) and micronutrient solution with composition in mg/L of (H_3BO_3:600; $CoCl_2$:400; $ZnSO_4 \cdot 7H_2O$:200; $MnCl_2$:60; $Na_2MoO_4 \cdot 7H_2O$:60; $NiCl_2$:40; $CuCl_2$:20) (Huang *et al*., 2012) were purchased from Merck, Mumbai, India. The materials like sterilized polybag, parafilm, micro-pipette and micro-pipette tube, append-off, silicon tube, cotton bundles, inoculation loop, were purchased from Gyan scientific Varanasi.

3.1.1 Equipments

The equipments used in the present work were incubator, hot plate, COD digester, autoclave, sonicator, sprit lamp, laminar flow, peristaltic pump etc

3.1.2 Glass Wares

Shake flasks, beakers, measuring cylinders, glass bottles, test tubes, volumetric flasks, petridishes, 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. Prior to use all the glass wares were sterilized by sulphuric acid and potassium dichromate and thereafter rinsed properly with distilled water.

3.2 Methods

3.2.1 Site description and soil sampling

Soil samples were collected in sterile polybag from the agricultural fields of Institute of Agricultural Science (BHU) Varanasi, India (25◦ 19' N; 83◦ 3'E, 129 m above mean sea level). The site was regularly exposed to Malathion by the agricultural scientist for destroying the insects and pests. The soil sample was taken to the depth of 20 cm using a soil corer of $1cm^3$. After sampling, the soil was sieved using 2 mm mesh for the removal of debris and coarse particles present in it and stored at 4^oC for further analysis (**Fig. 2**).

Figure 2: Soil incubation in Laboratory

Enrichment of soil samples was performed in Mineral Salt Medium (MSM) with the help of malathion as a sole carbon source. The composition of MSM (g/L) was K_2HPO_4 (800 mg/L), KH_2PO_4 (200); $MgSO_4 \cdot 7H_2O$ (500mg/L); $CaSO_4 \cdot 2H_2O$ (50mg/L); $(NH2)_2SO_4$ (1000mg/L) and ($FeSO_4$ (10mg/L) in distilled water (Rene *et al*., 2005). The MSM was sterilized by autoclaving for 20 min at 121°C, and final pH was maintained at 7.5 ±0.3, and then the sterilized MSM and pesticides was used for the isolation of potential microbes, the

plates are as shown in **Figure 3 (a)** and **(b)**. Further enrichment procedure was performed as described by Srivastva *et al*. (2016).

Figure 3 (a): Nutrient agar plating

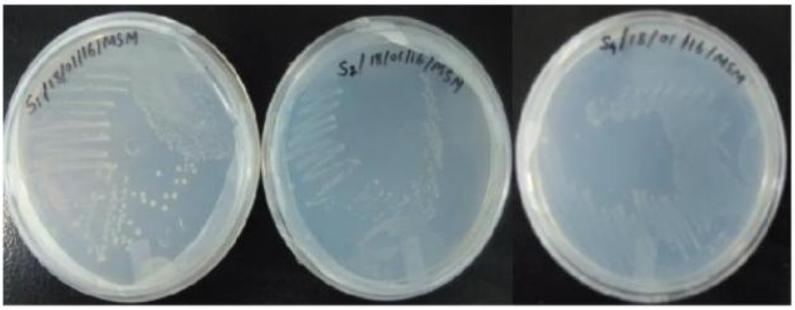

Figure 3 (b): MSM agar and pesticide plating

3.2.2 Packing media (PUF and Loofa)

PUF sheet of 1 cm thickness was purchased from Prakash Books Shop (Varanasi, India) for using as packing material in the bioreactor. The PUF sheet was cut into pieces of 1 cm^3 washed thoroughly with distilled water followed by ethanol. After washing PUF cubes were squeezed and dried overnight at 45 ºC and stored for biodegradation experiments. Loofa was procured from the local market in Varanasi, India.

The PUF and Loofa were immobilized with isolated bacterial species, 15 days ahead of biodegradation experiments to confirm decent growth of bacteria. SEM images corresponding to 0^{th} day, 15^{th} day (beginning of PBBR) and 75^{th} day (termination of PBBR operation) in the presence of Malathion were obtained for analysis. The **Figure 4** show that

the PUF has several microspores which are well interlinked to each other and thus provide a larger surface area for the development of a biofilm and enhanced percolation of media through the microporous structure.

PUF samples collected on the 0^{th} day showed no signs of bacterial growth, whereas rod-shaped bacterial species of uniform size were obtained on the PUF collected on the 15^{th} day. The results obtained were similar to the results of molecular characterization which concludes that the *Bacillus* sp. S4 (rod-shaped bacteria) was successfully acclimated on PUF which are dependent upon paramaters such as the adaptive capability of microorganism in a bioreactor, nutrient availability, Malathion concentration, etc. SEM image corresponding to 75^{th} day (termination of the operation) was fairly identical to image taken on the 15^{th} day which showed that *Bacillus* sp. S4 was capable of withstanding high and differing loading rates of Malathion, and hence there is little or no contamination with time. Identical results were shown on the morphology of PUF and microorganisms by several researchers for Malathion biodegradation with indigenous acclimated activated sludge (Tazdait *et al*., 2013).

Figure 4: Cubes of PUF used as a packing media

Loofa sponge was washed and cut into the size of 1 × 1.25 × 1.25 cm^3 (**Fig 5**). The Loofa sponge was characterized using similar methods as used by Shahi *et al*. (2016). The characterization results show that the Loofa sponge has a porosity of 72%, bulk density of 38 kg/m^3 and moisture holding capacity of 2.98 gm H_2O/gm of dry loofa. The batch experiments were conducted in screw cap bottles to study and evaluate the adsorption capacity of loofa for atrazine. Atrazine was taken in the screw cap bottles at two concentrations of 100 and 150 mg/L, closed with a cork. In both the bottles, loofa pieces were placed, and then the bottles were kept on a rotary shaker at 160 rpm for 72 hr. GC was used to measure the aqueous phase Atrazine concentration. The results revealed that loofa had almost negligible adsorption capacity for Atrazine. A detail experimental procedure was described in the literature Singh *et al.* (2010). The changes in Atrazine concentration were measured and were found to be insignificant. Further, study was performed in batch bioreactor to understand the potential of microbial species in reactor.

Figure 5: Pieces of Loofa sponge

The pieces of loofa were inoculated with an isolated culture of bacterial species for immobilized cell and packed batch bioreactor study.

3.2.3 Identification of bacterial species

Morphological analysis of bacterial species was performed using SEM (QUANTA 200F Netherland). The sample consisting of bacterial sp. was intially strained through the strips of the cellulose paper (Ultipor N66, Life Sciences, India). Further, the cellulose papers containing bacterium were dried overnight at 30^{o}C and the samples are coated by using gold particle to increase the conductivity of the sample to get better resolution. In order to diminish decay to the bacteria, the SEM analysis was performed under vacuum.

Biochemical kit (**Fig 6**) was purchased and used based on the guidelines mentioned in Bergey's manual of bacteriology to carry out biochemical tests- Catalase, oxidase, voges-proskauer, urease and fermentation reaction for the isolated microorganism (Geed *et al*., 2017). Grams staining were performed to sort bacteria into two broad categories, gram-negative and gram-positive. A heat-fixed smear coverslip was stained with crystal violet for one minute before washing with water. After being washed with water, the coverslip was covered with iodine for another minute. When the iodine was washed off both types of bacteria appeared dark violet. The coverslip was decolorized with 95% Ethanol to remove the purple from the cells of gram-negative species but not gram-positive species. The coverslip was carefully rinsed off, and stained with safranin, a basic red dye, for another minute. The dye was washed off with water. After air drying, the coverslip was ready to be examined under a laboratory microscope (Singh *et al.*, 2010).

Figure 6: Biochemical identification test kit

Bacterium genomic DNA was separated using the basic protocol of Sambrook *et al*. (2001). The details procedure is explained by Shukla *et al*. (2010). Genomic DNA was exposed 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; 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 50µL containing 10X buffer, 0.5mM $MgCl_2$, 2.5 Mm each dNTP, 1U of Taq DNA polymerase, each primer with a concentration of 10µM, along with 1µL of the DNA template. Each cycle comprised of an starting denaturation temperature at 94°C for 1 minute followed by 30 cycles of amplification program consisting of a denaturation step at 94°C for 50 seconds. Further, annealing was carried out at 60°C for 30 sec and heated upto a temperature of 72°C for 1.3 min and final extension temperature was maintained at 72 ^{o}C for 6 min. The PCR products run on 1% agarose gel. The gel has been coated with ethidium bromide, visualized under UV light and spot identification on gel.

Sequencing of the bacterial isolate was carried out by Triyat Scientific, Hyderabad, India. The sequences collected were used to evaluate the similarity of nucleotides to the available 16S-rRNA gene sequences in the Gene Bank database with the help of nucleotide BLAST program useable in NCBI (National Centre for Biotechnology Information) database. Additionally, the sequences were aligned manually with reported sequences in NCBI database by using CLUSTALW multiple sequence alignment program. For circular comparative analysis of selected homologous genes along with targeted *Bacillus* sp. S_1, *Bacillus* sp. S_2 and *Bacillus* sp. S_4 was done with Cicos (Krzywinski et al., 2009).

3.2.4 Selection of best bacterial isolate for malathion degradation

The free cell batch experiments were performed for three bacterial isolates at Malathion concentration ranging from 25-700 mg/L with an increase of 25 mg/L. For the biodegradation study, all the species were initially precultured to maximize the bacterial density, cell suspensions with the volume of 500µl were inoculated with 100mL MSM medium in 120 mL of serum bottle of different concentration. A control was prepared in a serum bottle free cell of bacterial inoculums bearing the same Malathion concentration respectively. The incubation was carried out at 32^0C on a shaker at 150 rpm for 10 days. All the trials were performed in triplicate to minimize the experimental error. Remaining Malathion concentration from the bacterial media was obtained using a same known volume of chloroform and HCl in a separating funnel. The concentration of the residue was analyzed by GC-FID, at an interval 24h upto10 days of incubating periods respectively.

The mean Malathion degradation rates (average) forvarying substrate concentration were calculated using the equation (1)

$$r_{ave(d^{-1})} = \frac{C_0 - C_t}{C_o(\Delta t)} \qquad (1)$$

C_o and C_t are the substrate concentrations at time t = 0 and t, respectively.

r_{avg} shows the average rate of degradation and Δt shows degradation time.

The growth and inhibition kinetic constants were estimated with the help of Monod and Andrew-Haldane model corresponding to which maximum degradation rates were observed.

3.2.5 Effect of pH and temperature on free cell biodegradation

The effect of process parameters such as pH (range5-10) with an increment of 0.5 and temperature (24-44^{o}C) with an increment of 2^{o}C on biodegradation were performed at fixed Malathion concentration of 150 mg/L. Further, the experiment was performed at optimized pH and temperature by varying the Malathion concentrations (25-700 mg/L) to optimize the concentration in the free cell. Optimized conditions were used in further experiments. The bacterial maturation was checked on a regular basis using a Elico UV-VIS spectrophotometer Model no SL-159 at a wavelength of 600 nm. All trials were conducted in triplicate to minimize the experimental error.

3.2.6 Biodegradation of Malathion in the packed batch bioreactor

The bioreactor was fabricated borosilicate glass having a length of 55 cm, the internal diameter of 6.5 cm with a 1000 mL working volume and total volume 1824 mL used in batch and continuous mode. The inlet and outlet ports were provoide to charge and discharge of feed and residue respectively. All the openings of the sampling ports were closed using silicon tubing with pinch cork. The supply of air for maintaining oxic condition through the bed was done by compressor (KHOSLA, India S.N.65739). The flow rate was controlled by rotameter. Air was introduced just 2.5 mm above the bottom through sparger which also caused liquid circulation. The experimental setup is shown in Figure 7 and dimension details are listed in **Table 4** which was similar for both batch and continuous bioreactor. Loofa

sponge was used as a packing media. The Loofa pieces were washed twice with distilled water and ethanol alternatively, dried overnight at 45 °C and cooled in a desiccator. These dried cubes were used as packing material for biodegradation experiments in the bioreactor. Loofa was immobilized with *Bacillus* sp. S_4 for 20 days before starting the biodegradation experimental trials. The Malathion concentration 200 mg/L was added into bioreactor at pH (7.5±0.3) and room temperature (25±^{o}C) for Malathion degradation and performance of bioreactor.

3.2.7 Operation of continuous bioreactor

In a continuous bioreactor the PUF sheet of 1 cm thick was utilized as packing media. The PUF sheet was sliced into small parts of 1 cm^3 and then washed carefully with distilled water and the using ethanol dried and stored over night at 45^{o}C.

The batch experiments were conducted in screw cap bottles to estimate the adsorption of Malathion on PUF. Firstly, the PUF pieces were put into the bottle and then prepared Malathion solutions of concentration 150 and 250 mg/L were filled into a screw cap bottle and sealed using a cork. PUF pieces were added into both the bottles and then the sealed bottles were placed on a shaker at 150 rpm for 84 h.

Malathion concentration was measured in both aqueous phase as well as head space of both the bottles at fixed intervals using gas chromatography. The result revealed that PUF has almost negligible adsorption capacity for Malathion. The details of the experiments were given by Singh *et al*. (2010).

Table 4: Dimensional details and performance of bioreactors at optimal operating conditions for biodegradation Malathion

Parameters	**Value**
Height of the packed bed (cm)	45
Diameter of reactor (cm)	6.5
Total volume (mL)	1824
Working volume (mL)	1000
Void volume (mL)	824
Packing material	PUF
Packing material size (cm^3)	1
Ph	7.0±0.2
Temperature (^oC)	33±5
DO (mg/L)	6
Inlet concentration (mg/L)	300
Loading rate (mg/L/day)	36-216
Outlet concentration (mg/L)	21
EC (mg/L/day)	7.20-145.4
RE %	>90
Solution treated (L)	400
Operation Time (day)	75
Microbial growth (g/L)	-

3.2.8 Batch bioreactor and parametric optimization

The process optimization was done using a set of variable process parameters like pH, temperature and DO. Experiments were carried out in a batch (flask) using 125 mg/L Malathion solution. Optimization was done by changing a single parameter at a time keeping remaining parameters constant. During the first set of experiments the pH was changed from 5.0–10.0 at a constant temperature (37^oC), DO (6.0 mg/L) and Malathion concentration of 125 mg/L. The result of pH optimization was used to optimize temperature. Likewise DO was also optimized. Further, at optimum pH, temperature and DO the experimental trials were carried out by changing Malathion concentration from 50-1000 mg/L to evaluate the relation of inlet Malathion loading rate on % removal elimination and study the substrate

inhibition outcome on biodegradation rate. All experimental trials were conducted in triplicate to decrease the experimental error.

3.2.9 Continuous study

Packed Bed Bioreactor (PBBR) (**Fig. 7**) was administrated in a continuous mode using optimum parameters estimated previously from the batch reactor study. The PUF was immobilized with *Bacillus* sp. S_4, 15 days ahead of biodegradation experiments to confirm decent growth of bacteria. The Malathion consisting of feed solution of concentration 300 mg/L was prepared and collected in a 100 L vessel (feed solution tank) and sent into the continuous PBBR with the help of a peristaltic pump (McLins PP10) at flow rate of 5–35 mL/h. Nutrients and filtered air were supplied through the provisions made to the bioreactor for controlling required DO level. All the values were recorded in the duplicate at a regular interval of time, and mean value was selected for interpretation and analysis. The continuous PBBR was regulated for 75 day, and its performance was studied with the help of following parameters:

$$\text{Elimination capacity (EC)} = Q\,\frac{(S_{in} - S_{out})}{V} \quad (2)$$

$$\%\text{Removal efficiency (RE)} = \frac{\boldsymbol{S_{in} - S_{out}}}{\boldsymbol{S_{in}}} \times 100 \quad (3)$$

$$\text{Inlet loading rates (IL)} = \frac{S_{in}}{V}\,Q \quad (4)$$

where S_{in} and S_{out} shows inlet and outlet concentrations of Malathion in the bioreactor, Q shows volumetric flow rate and V shows hold-up volume of the reactor.

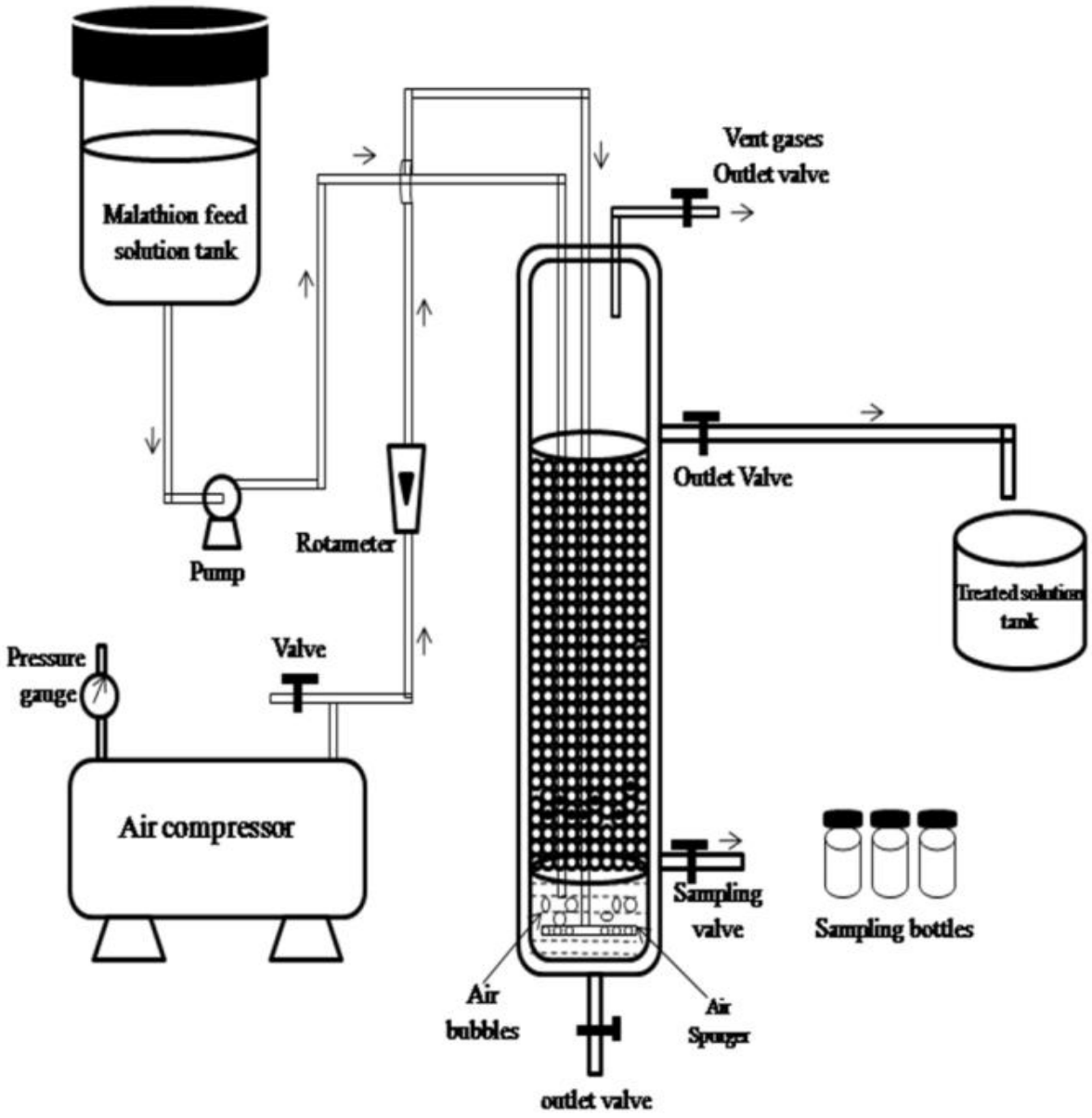

Figure 7(a): Schematics of packed bed bioreactor system

Figure 7(b): Laboratory experimental setup of packed bed bioreactor system

3.3 Malathion biodegradation: growth and inhibition kinetics

The growth and kinetic inhibition parameters were estimated in free cell system by considering parameters including bacterial growth, substrate concentrations and time. The Monod growth kinetic model was preferred to fit experimental data in this study. The Monod model equation is given as

$$\mu = \frac{1}{X}\frac{dx}{dt} = \frac{\mu_{max} S}{K_s + S} \qquad (5)$$

Where, μ shows specific growth rate (1/day), μ_{max} shows maximum specific growth rate (1/day), K_s shows half-saturation constant (mg/L), X shows microbial cell, S shows initial substrate concentration (mg/L), and *t* shows time. Linear form of equation (6) was

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}\, S} + \frac{1}{\mu_{max}} \qquad (6)$$

The value of $\frac{1}{\mu}$ and $\frac{1}{S}$, were calculated and plotted to obtain μ_{max} and K_s.

Higher Malathion concentration inhibits bacterial growth. In such condition, Monod kinetic model was unable to predict inhibition, hence, Andrews-Haldane model (Haldane 1930; Andrews 1968) was used for the calculation of kinetic parameters including inhibition constant K_i (mg/L). The inhibition kinetics equation is given below

$$\mu = \mu_{max} \frac{S}{K_s + S + \frac{S^2}{K_i}} \qquad (7)$$

3.4 Proteomics study for malathion degradation

3.4.1 Extraction, quantification and 2-Dimensional electrophoresis separation of proteins

The cells of *Bacillus* species treated with malathion and control were centrifuged (10,000× g, 10 min). The pallets were separated and mixed with 2 mL extraction buffer consisting of 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$ and 10 mM KCl additionally 10 μM PMSF(phenylmethylsulphonyl fluoride). The suspension was ground wth liquid nitrogen and again centrifuge at 12,000× g for 1 h. Extraction method as described by Singh et al. (2015) was used. The immobilized pH gradient (IPG) strip was installed on the top of 12.0% SDS-PAGE and sealed with 0.5% agarose. Electrophoresis was completed (**Fig 8**) through a10 mA/gel for 30 min followed by 25 mA/gel for 5 h operated in a Hoefer SE 600 apparatus (Amersham Biosciences, USA). For 2DE gel image analysis PDQuest™ 8.0.1(Bio-Rad)

software was used for the gel to gel matching, spot detection, quantification (as spot volumes, intensity × mm^2), identification of differences in spot intensities, background subtraction, and spot matching between treated and untreated/control sample. Missing spot volumes were estimated with the help of values engaging a sequential K-nearest neighbor algorithm using R 2.7.0 environment. Estimating missing value imputation, total spot volume per gel was correlated to normalize spot intensities (% of individual spot volume/Σ% spot volume of each gel) and to take care of variations between gel replicates. The data represented was cubic root transformed to minimize the spot volume-spot deviation dependency. Overall 275 protein spots were recognized from the gel having a statistical significance (p <0.05) and reproducibility with an abundance ratio of at least 1.5 fold was taken into consideration as differentially expressed proteins.

Figure 8: Gel images of 2D gel electrophoresis

3.4.2 Identification of protein: MALDI-TOF MS/MS Analysis

The samples for MALDI-TOF analysis were processed as per the procedure mentioned by Bruker Daltonics (Shevchenko *et al*., 2007). The protein digestion protocol was followed as given by Singh *et al*. (2015). Extracted peptides were vacuum concentrated. 1µL of concentrate peptides was treated with trypsine and applied on CHCA(α-cyno-4-

hydroxycinnamic acid) matrix (2.5 mg/mL in 50% acetonitrile) on the target plate (400well 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 v 2.4 was used for protein database searches. The data was searched against the NCBI (The National Centre for biotechnology information).The data for BLAST searches comprised of groups of single spot peptide sequences found with the help of Data Explorer software (AB Sciex, USA).

The analysis was carried out on an AB Sciex 5800 TOF/TOF^{TM} system (AB Sciex, USA). Samples were applied on the MALDI plate 5 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 watts/m^2 and pulse rate of 400Hz. The precursor selection was filtered from strongest to weakest in this mode. The instrument in TOF MS mode was initially evaluated externally and then internally for every sample with 842.510 m/z and 2211.106 m/z (trypsine autolytic peptides).

3.5 *In silico* analysis and characterization of identified protein

3.5.1 Functional domain analysis, phylogenic classification and structural prediction

Functional domain analysis is a technique used to assign the biological or functional role. The PROSITE server was used for functional domain analysis. PROSITE is a method of identifying the function of uncharacterized proteins. It consists of a database of biologically significant patterns and profiles formulated in such a way that with appropriate computational tools it can rapidly and reliably determine to which family the particular protein sequence belongs (Hofmann *et al*., 1999).

In phylogenetic analysis, multiple sequence alignment was performed to identify well conserved and divergent regions and aligned positions. Phylogenetic method eliminates such positions and at the same time tries to minimize the loss of informative sites (Castresana, 2000). Phylogenetic analysis is based on the selection of blocks of positions that will fulfill a simple set of requirements concerning the number of contiguous conserved positions, high conservation of flanking positions and lack of gaps for making the final alignment (Castresana, 2000). Clustalw performed the close and even relationship among the selected homologous genes for phylogenetic and sequential classification based on multiple alignment sequence, for phylogenetic contraction MEGA6 tool was used (Thompson *et al*., 1997).

The structural prediction of a protein is correlated with its amino acid composition and was performed for characterization of any hypothetical and identified proteins using homology modeling (Vyas *et al*., 2012). Homology models of proteins show considerable interest in planning and analyzing the biological experiments. Homology modeling was done to construct the three-dimensional protein structures using atomic resolution template structure (Vyas *et al*., 2012). The structure was predicted by sequence identity > 20%, below 20% sequence identity can have the very different structure (Krieger *et al*., 2004; Vyas *et al*., 2012). RAMPAGE (Ramachandran Plot) was used for quality assessment, and structural classification was performed by CATH server (Lovell *et al*., 2003). Out of the 7 proteins, the good quality model was taken NP_390682.1 (Hypothetical protein) for docking calculation. Molecular docking analysis was performed using YASARA (Yet Another Scientific Artificial Reality Application) (Krieger *et al*., 2004)

3.5.2 Active site analysis and Docking

Active sites were analyzed using meta pocket server (Huang, 2009) and the best predicted model was taken for docking calculation. Molecular docking was performed using YASARA. Further, each docking complex was visualized using discovery studio 3.0.

3.6 Integrated two stage bioreactor study for mixed pesticides

3.6.1 Experimental setup for treatment of mixed pesticides: reactor setup and operations

The two-stage Integrated Aerobic Treatment Plant (IATP) (TAE/1000 Pignat, France) with working volume of 30 liters and total volume of 45 liters was used for the treatment of synthetic wastewater. The synthetic wastewater having 50 mg/L each of Atrazine, Malathion and Parathion were chosen to optimize concentration in batch incubation study changing concentration from 25-100 mg/L. The COD value of (50 mg/L) prepared synthetic wastewater was measured as 1232 mg/L. A 200 L capacity plastic tank was used to store synthetic waste. The first stage in the integrated aerobic treatment plant was stirred aerobic bioreactor (Reactor-1) as demonstrated in **Figure 9**. The stirring was carried out by motor-1 (IKA, Eurostar 40) with Rushton turbine rotating continuously at 160 rpm, along with air which is sent through an air compressor (KNF, Labport). The timer was fixed for a period of 30 min/hr to control proper aeration in the reactor. Various specifications such as pH, Redox potential and DO (HD 2305.0; Delta OHM; Italy) were evaluated using respective probe which was procured from Pignat, France.

Firstly, the extracted bacterial species was cultured in Nutrient broth was placed in 100 mL screw cap bottles. The freshly prepared bacterial biomass was cultured with glucose (100 g/L) alongwith MSM and trace element solution in the Reactor-1 and maintained at 240 hr

(10days) for proper growth of bacterial species. After 240 hr, COD of synthetic wastewater (1232 mg/L) was fed into Reactor-1. Every day during the treatment process, 10L fresh wastewater (limited feeding; 2 hr feed inlet and 10 hr treatment every day) was fed to the Reactor-1 for proper performance and treatment of synthetic wastewater in reactor.

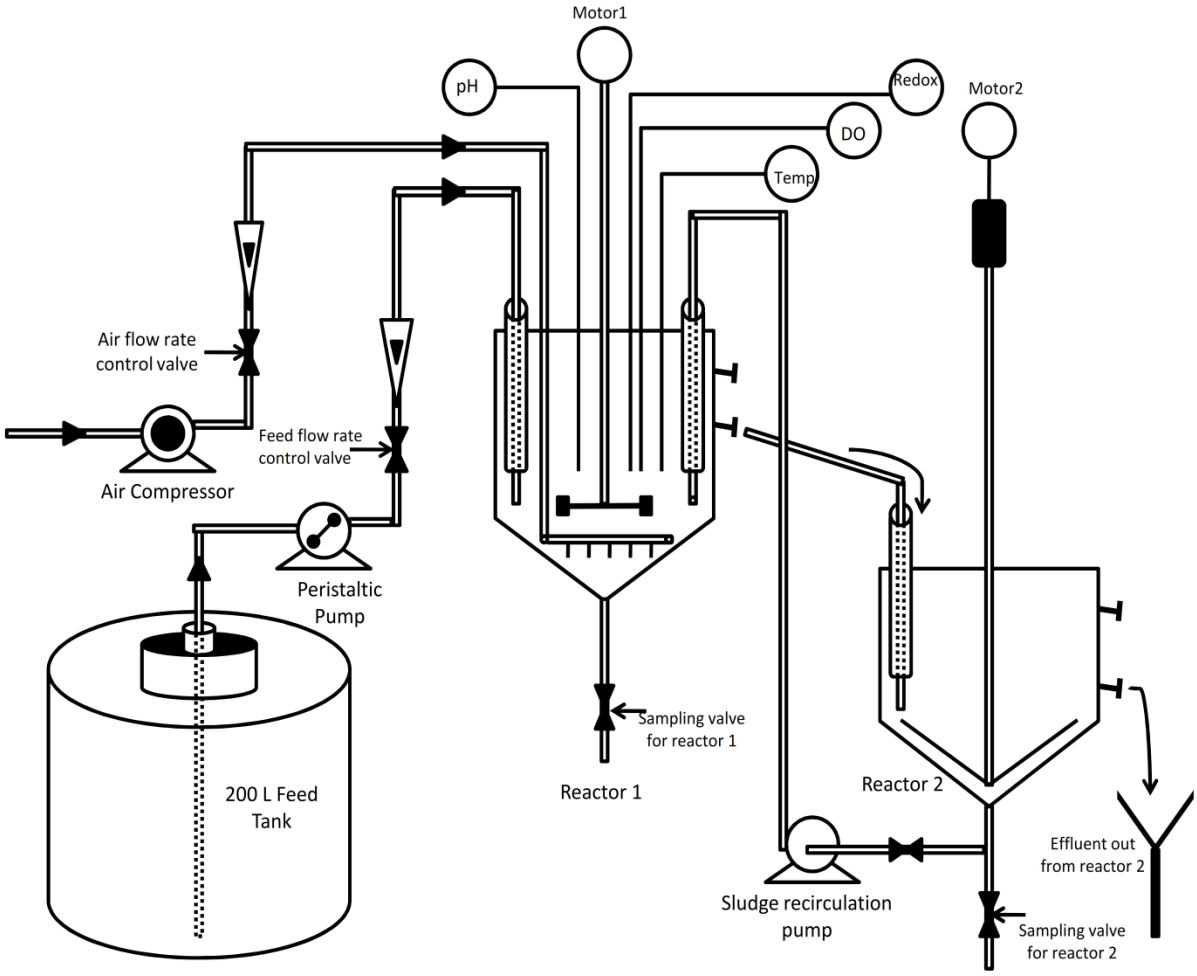

Figure 9: Schematics of two stage integrated aerobic treatment plant

The treated effluent of Reactor-1 was sent into Reactor-2 (second stage; settling tank) consisting of stirring motor-2. In Reactor-2, the residual parental pesticides and metabolites were treated with part of effluent recycled from Reactor-1 using a recycle pump (KNF, Liquiport) which was maintained at constant flow rate of 6 L/h. The samples for further

analysis were withdrawn in duplicate at an regular interval of 12 h for 30 consecutive days from the sampling valve of reactor-1 and reactor-2. The variables such as pH, Redox, DO, COD and COD/BOD5 were recorded. The ANOVA analysis was performed to estimatethe significance of pH, DO, Redox and BOD5/COD ratio on COD removal. The COD removal was using equation (8)

$$\%\ \text{COD Removal} = \frac{\text{COD}_{\text{initial}} - \text{COD}_{\text{final}}}{\text{COD}_{\text{initial}}} \times 100 \qquad (8)$$

3.6.2 Kinetic study

Bailey and Ollis (1986) communicated various models relating to growth and substrate utilization rate with the feasible substrate concentration. After referring a few literature reports, the Monod model was widely acceptable for mathematical representation of the growth of microorganism in such systems (Monod, 1949). The model equations are given by

$$\mu = \frac{1}{X}\frac{dx}{dt} = \frac{\mu_{max} \cdot C}{K_s + C} \qquad (9)$$

By simplifying equation (9) get

$$\frac{dx}{dt} = \mu_{max}\frac{X \cdot C}{K_s + C} \qquad (10)$$

Where μ shows specific growth rate/hr; μ_{max} shows maximum specific growth rate/hr; C shows substrate concentration (mg/L) and K_s shows half saturation rate constant (mg/L).

In biological processes, $r_g=(dx/dt)$ the growth ratecanbe defined as (11)

$$r_g = \mu \cdot X \qquad (11)$$

Where r_g is the rate of bacterial growth, mg/L/hr, and X represents microorganism dry weight cell mass (mg/L).

Monod model can be fitted to the equation, the substrate concentration (Ks >> C) (Beltran-Heredia *et al*., 2000)

$$\frac{dx}{dt} = -K_{obs} \cdot C \qquad (12)$$

Where K_{obs} shows first order rate kinetic constant (per day)

As $C_o > 0$, the differential equation was evaluated to get the following equation

$$Ln\frac{C_0}{C_t} = K_{obs} \cdot t \qquad (13)$$

Where C_t and C_o (Chemical Oxygen Demand mg/L) are the residue concentration of substrate at time t (day; end of cultivating time) and the initial concentration respectively; K_{obs} (per day).

A plot of $Ln(C_t/C_o)$ *vs*. time t (day; end of cultivating time) as demonstrated and the value of constant K_{obs} and correlation coefficient R^2 were measured.

Cell yield coefficient ($Y_{x/c}$) and biomass decay coefficient (K_{dp}) are essential parameters associated to the biomass concentration expansion till terimination of the aerobic treatment plant. The $Y_{x/c}$ indicates the mass of biomass produced per mass of substrate taken, and K_{dp} represents the significance of endogenous metabolism. The $Y_{x/c}$ and K_{dp} were measured using specific growth rate equation (Beltran-Heredia *et al*., 2000) as shown

$$\mu = Y_{\frac{X}{C}} \cdot q - K_{dp} \qquad (14)$$

Where, μ shows specific growth rate/ hr; q shows specific substrate utilization rate; *Yx/c* shows ratio of the mass of cell generated to the mass of substrate taken; and K_{dp} shows kinetic constant for biomass death phase/hr (Affam *et al*., 2014). μ is measured by equation (15) and q is measured by equation (16).

$$\mu = \frac{1}{X}\frac{\Delta X}{\Delta t} \qquad (15)$$

$$q = -\frac{1}{x}\frac{\Delta C}{\Delta t} \qquad (16)$$

3.7 Hybrid bioreactor study for atrazine removal

3.7.1 Isolation of Atrazine degrading microbes

Atrazine containing soil samples were collected from Banaras Hindu University's agricultural farm, Varanasi, India (25◦19'N; 83◦3'E, 129 m above sea level). Atrazine is one the most commonly used pesticide in agricultural farm at BHU. Thus soil containing atrazine is readily available at selected place. The enrichment of isolated bacterial consortia was completed using MSM and Atrazine as exclusive carbon source. The morphology of consortia was carried out SEM (SEM QUANTA 200F, Netherland). The Loofa samples accumulated with bacterium was sliced into pieces, dried overnight at 30 ^{o}C in the furnace (Model 159SS NSW, India) and finally embedded with gold and carbon particles for better resolution. The SEM analysis was carried out at low pressure (Thomas et al., 2015).

3.7.2 Parametric optimization in batch process

3.7.2.1 UV Fenton

All the experiments were carried out in 1 L glass reactor as shown in **Figure 10** at room temperature (23±3 ºC) and stirred at 200 rpm for 1 hr. The parametric effects such as mole ratio of H_2O_2/COD (0.25-1.5), H_2O_2 /Fe^{2+} (5-50) and pH (1.0–5.0) on UV-Fenton reaction were studied in the Fenton reactor to measure the optimum condition maintaining the Atrazine concentration at 150 mg/L. The response of H_2O_2/COD molar ratio was examined by varying the ratio from 0.25 to 1.5 keeping the other operating parameters fixed (pH 2.5, H_2O_2 /Fe^{2+} 25, initial COD 2900 mg/L, reaction time 60 min and 200 rpm).

Similarly, the effects of pH and H_2O_2/Fe^{2+} molar ratio on the removal of Atrazine were studied simultaneously by varying molar ratios from 5 to 50 and pH from 1 to 5.All experimental trials were recorded in triplicates to decrease the experimental error. The

optimum process parameters were used for the removal of Atrazine. Experiments were performed under optimum operating conditions to obtain the maximum removal by UV Fenton process to degrade Atrazine.

3.7.2.2 Biological process: bioreactor

The schematic diagrams of the bioreactor are shown in **Figure 10** with all the outlet sampling ports were closed during the experimental period. The experiments were conducted to study the parametric effects such as pH (4.0 to 9.0) and DO (3.5 to 7.5mg/L) on biodegradation of Atrazine. pH and DO were varied, maintaining the Atrazine concentration at constant value of 150 mg/L to get their optimum values. Effect on Atrazine concentration was estimated at an optimized amount of pH and DO by changing the Atrazine concentration in the range of 50-500 mg/L. All experimental trials were recorded in duplicate to decrease the error.

3.7.3 Experimental set-up

The experimental setup of coupled system is as shown in **Figure 10**. The experiments were carried out in a 1000 mL reactor filled with 500 mL of the sample mixed at 200 rpm using magnetic stirrer (ELICO; GI 631) at room temperature (23±3 ºC) in three different stages, where stage-I was UV Fenton process. The pH (2.5) of the solution was maintained by flow controller using 1N HCl and 1N NaOH. Afterward, $FeSO_4$ salt 206g/L and 2.1 ml H_2O_2 (30%w/v) were added to the reactor. Then the experimental solution was irradiated with a UV Lamp (Mineralight; V41) for a reaction time of 60 min at 200 rpm. The Fenton effluent was filtered using filter paper (Ultipore N66, Pall Life Sciences, India) with vacuum filtration Unit (Riviera Glass Pvt Limited, India).

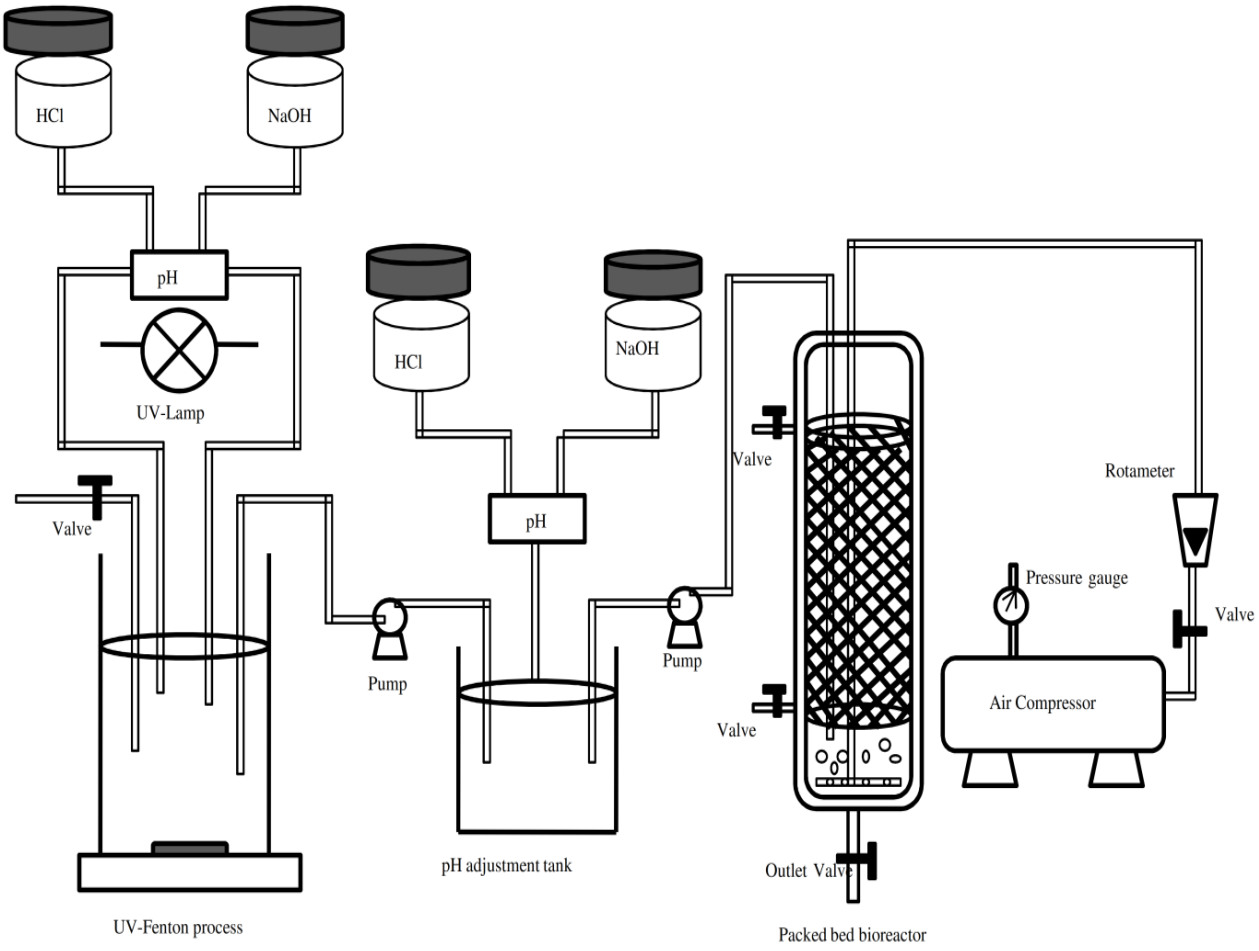

Figure 10: Schematic diagram of coupling method UV-Fenton-Bio-reactor in series

Stage-II consisted of 1-liter stabilization reactor. The effluent from UV-Fenton was fed to the stabilization reactor. The effluent from UV Fenton was filtered, and pH was adjusted to neutral (7±0.2). Finally, this effluent from stabilization was fed to the bioreactor (stage-III) for biodegradation of Atrazine at room temperature (23±3 ºC). The bioreactor was made of borosilicate glass of length 55 cm, internal diameter 6.5 cm of 1000 mL working volume (1826 mL; actual volume) as shown in **Figure 10**. Loofa purchased from the local market Varanasi, Uttar Pradesh, India was used as a packing material for the treatment of Atrazine. The inlet and outlet ports were equipped to aid entry and execution of feed/residue.

The outlet was fixed with 0.2 µm filter to stop the exit of bacteria from the bioreactor. Silicon tubing was used to close all the sampling ports plus pinch cork. Air was sent through the compressor (Khosla, India S.N.65739) to the bioreactor to control the aerobic state and proper mixing. The aeration rate was maintained using a pre-calibrated rotameter (Eureka, Pune, Model SRS-MG5) with a flow rate of 5-35 L/min. Air sparger was used for uniform distribution of air and was placed 2.5 mm above the base of reactor upward direction to maintain the liquid in agitated state.

3.8 Treated effluent analysis

3.8.1 Malathion and its metabolites analysis

Malathion concentrations were analyzed with the help of GC having a flame-ionization detector (FID) (Thermo scientific, trace 1110) along with a BP-5 capillary column of dimensions 25m×0.32mm. The samples were prepared using extracted residual Malathion along with an same amount of chloroform and degraded sample. The organic layer was separated by shaking the contents vigorously. The oven was maintained at a temperature of 90 °C initially for 1 min and then heated upto 170 °C at a rate of 10 °C/min for 1 min and again up to 200°C at a rate of 10 °C /min amidst overall running time of 15 min. Temperature of injector and detector were controlled at 220 °C and 230 °C respectively (Geed *et al*., 2016).

The samples were collected after 10 days of degradation experiment to know the intermediate products formed during the biodegradation. The organic fractions present in the sample were extracted using chloroform as solvent after removing the cell biomass through centrifugation. The extracted organic fraction was analyzed using FT-IR (NICOLET 5700 FT-IR, Japan) to know the functional groups of the extracted organic fractions.

The extracted samples were analyzed to know the presence of different organic intermediates/compounds present in it with the help of GC-MS-QP2010 Ultra (Shimadzu, Japan) equipped with flame ionization detector, using a capillary column whose flow rate was regulated at 1.21 mL/min with a split ratio of 100 and injection temperature of 260°C. Further, conditions for MS measurements were MS ion source at 230 °C, MS interface temperature of 270°C, totaltime period of 20 min, solvent delay of 3 min, chromatographic data were obtained by GC-MS (Singh *et al*., 2012).

3.8.2 Effluent analysis of IATP for mixed pesticide treatment

COD and BOD of firstly prepared synthetic wastewater and treated samples were estimated accordingly with the standard procedure (APHA, 2005) carried out in the laboratory. The physico-chemical specifications of wastewater were analyzed by Ion Chromatography (Dionex ICS 3000).

HPLC (ELICO, India) installed with C-18 column, ultraviolet detector (HD469) and pump (HD464) was used to analyze the concentration of residue mixture of pesticides and their metabolites at a wavelength of 210 nm with the help of mobile phase (acetonitrile:water; 45:55) for 15 min with a constant flow rate of 1.0 mL/min (Douglass *et al*., 2014).

For GC-MS analysis samples were made by extracting residual metabolites of pesticides using same volume (5 mL) of chloroform and degraded sample. The organic layer was separated from the prepared samples through vigorous shaking of the vials. The extracted samples were analyzed to know the presence of different organic compounds present in it using GC-MS-QP2010 Ultra (Shimadzu, Japan) equipped with flame ionization detector, using a capillary column whose flow rate was set at 1.21 mL/min with a split ratio

of 10. The furnace was held at 50 ^{O}C for 2 min and then heated at 5 ^{O}C/min to 250 ^{O}C, where it was holded for 2 min. Further, increment was done at the rate of 15 ^{O}C/min to 280 ^{O}C, where it was set to 14 min. Injector temperature was set to 260°C, and the oven temperature was set to 280 ^{O}C. For spectroscopy ion source was maintained at 230 °C, interface temperature at 270 °C, for duration of at 20 min, solvent delay for 3 min and chromatographic data were recorded by GC-MS (Singh *et al*., 2012).

3.8.3 Effluent analysis of hybrid bioreactor for atrazine removal

Chemical oxygen demand (COD) was determined according to the standard methods (APHA, 2005). To eliminate the interference of H_2O_2 and Fe^{2+} salt in COD analysis, samples were adjusted to pH 10 and stirred at 120 rpm for 30 min (Kang *et al*., 1999).

The residual Atrazine concentration and intermediate products from Fenton oxidation and biological processes were analyzed using HPLC (ELICO, India) having C-18 column and ultraviolet detector (HD469). Atrazine and by-products were determined at awavelength of 210 nm using mobile phase (acetonitrile:water; 45:55) for 15 min, maintained at constant flow rate of 1.0 mL/min (Alekseeva *et al*., 2011; Douglass *et al*., 2015).

GC-MS analysis was performed to confirm the presence of metabolites formed during Atrazine degradation. The samples were made using residual Atrazine with the inclusion of a same volume of Chloroform and degraded sample. The organic layer was removed by shaking the sample (Fang *et al*., 2015). The removed samples were analyzed with the help of GC-MS-QP2010 Ultra (JNU New Delhi). The gas flow rate was maintained at 1.21 mL/min with a split ratio of 10 at an injection temperature of 260 ^{O}C. Mass spectroscopy measurements were evaluated at a temperature of 230 ^{O}C using ion source at 270 ^{O}C for mass

spectroscopy interference, total time was maintained at 20 min with solvent delay for 3 min. Statistical analysis was performed by ANOVA.