CHAPTER 3

MATERIALS AND METHODS

3.1 GENERAL

This chapter gives the methodology during the selection and enrichment of the bacterial consortium from saline environment. The bacterial consortium was studied for degradation of selected phenolic compounds. The various conditions affecting growth of bacterial consortium like pH, salinity, nitrogen source and the incubation period were optimized for effective degradation of phenolic compounds. Degradation of selected phenolic compounds at various concentrations was studied at optimum salinity. The bacterial strains present in the consortium were identified by biochemical and molecular methods. The batch studies were conducted on degradation of phenol at different salt concentrations. The dioxygenase enzyme activity was assayed during the degradation of selected phenolic compounds. Bioreactor studies were conducted on treatment of phenol-laden saline wastewater. An overview of methodology used in the present study is schematically depicted in Figure 3.1.

3.2 CLEANING OF GLASSWARE

Glassware were first soaked in concentrated hydrochloric acid for a few hours and washed thoroughly in tap water. The glasswares were further washed with detergent, rinsed with distilled water and air-dried.
3.3 **STERILISATION**

The media were sterilized at 121°C for 15 minutes at 15 lbs/sq.in. pressure in an autoclave. Glassware were sterilized in a hot air oven at 170°C for 1 hour.
3.4 CHEMICALS

The phenolic compounds namely phenol, o-Cresol, m-Cresol, p-Cresol, 2-CP (2-chlorophenol), 4-CP (4-chlorophenol), 2,4 DCP (2,4-Dichlorophenol) were purchased from Merck, India (98% purity). 2,4,6 TCP (2,4,6-Trichlorophenol), Catechol and 3-chlorocatechol was purchased from Sigma Aldrich Chem. Co., USA (98-99% purity).

All other chemicals used in the mineral salt medium preparation were of analytical grade and purchased from Merck, India. Reagents and Enzymes used for molecular microbial studies were purchased from Qiagen (Germany) and Invitrogen (U.S.A).

3.5 MEDIA

(a) Mineral salts medium (MSM) (Alva et al. 2003)*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Borax</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>0.0125 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.06 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>30.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4±0.2</td>
</tr>
</tbody>
</table>

* The medium was slightly modified by removing the trace element solution and was added with or without yeast extract (0.1 g/L) and required amount of sodium chloride was added to the mineral salts medium, concentration from 10 g/L to 150 g/L, adjusted to pH -7.
b) **Nutrient agar (NA) medium**

- Peptone - 5.0 g
- Sodium chloride - 5.0 g
- Beef extract - 1.5 g
- Agar powder - 15.0 g
- pH - 7.2 ± 0.2
- Distilled water - 1 L

## 3.6 PHENOLIC COMPOUNDS

Phenolic compounds used in the study were grouped as Phenols: Phenol, o-Cresol, m-Cresol, p-Cresol, and Chlorophenols: 4-chlorophenol (CP), 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TCP). The properties of phenolic compounds used in the study are given in Table 3.1 and their structures are shown in Figure 3.2. Various concentrations of the phenolic compounds were prepared from the stock solutions as shown in Table 3.3.

### Table 3.1 Properties of phenolic compounds used in the study

<table>
<thead>
<tr>
<th>Properties</th>
<th>Phenol</th>
<th>o-cresol</th>
<th>m-cresol</th>
<th>p-cresol</th>
<th>4-CP</th>
<th>2,4-DCP</th>
<th>2,4,6-TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>C₆H₅OH</td>
<td>C₇H₈O</td>
<td>C₇H₈O</td>
<td>C₇H₈O</td>
<td>C₆H₂ClO</td>
<td>C₆H₂Cl₂O</td>
<td>C₆H₃Cl₃O</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>41</td>
<td>30.90</td>
<td>11.5</td>
<td>34.8</td>
<td>45</td>
<td>44</td>
<td>69</td>
</tr>
<tr>
<td>Boiling point(°C)</td>
<td>182</td>
<td>191</td>
<td>202.7</td>
<td>201.9</td>
<td>220</td>
<td>210</td>
<td>246</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>1.071</td>
<td>1.047</td>
<td>1.034</td>
<td>1.037</td>
<td>1.3</td>
<td>1.38</td>
<td>1.675</td>
</tr>
<tr>
<td>Solubility (25 °C)g/L</td>
<td>8.7</td>
<td>2.5</td>
<td>1.9</td>
<td>1.9</td>
<td>28.5</td>
<td>0.05</td>
<td>Nil</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>94.11</td>
<td>108.14</td>
<td>108.14</td>
<td>108.14</td>
<td>128.5</td>
<td>163.00</td>
<td>197.45</td>
</tr>
</tbody>
</table>
Figure 3.2 Structure of phenolic compounds used in the study

3.7 SCREENING OF BACTERIAL CONSORTIUM FROM ENVIRONMENTAL SAMPLES

3.7.1 Sample Collection

Soil samples were collected from six different habitats in Chennai (India) such as salt pans (Kelambakam), Puliket marine back water lake; Sea harbour (Chennai), phenol affected soils (phenol-resin manufacturing industry), tannery affected soils (Tannery industry) and soil from sea food industries.
3.7.2 Enrichment of Bacterial Consortium

Ten grams of soil collected from six different places were mixed together with 100 mL of sterilised distilled water containing 50 mg/L of phenol in mineral salts medium with 30 g/L NaCl concentration. Phenol was added from the stock solution to 250 mL Erlenmeyer flasks containing 100 mL of MSM and was incubated in rotary shaker at 150 rpm at incubation temperature of 37°C. Bacterial motility was checked with microscopic observation (Light Microscope). After the enrichment, samples were subjected to serial dilution using sterile distilled water and were pour plated with nutrient agar medium. The plates were incubated at room temperature for 48 h and the viable colony count was performed. At the end of fifth day, when the cell count reached 10⁷ cfu/mL, the culture was enriched several times after every five days. Increase in cell count from 10⁴ to 10⁹ cfu/mL on phenol as sole carbon source was taken as a confirmation for the ability of the consortium to utilize phenol. All the experiments were conducted in duplicates. The consortium was maintained on mineral salts medium with phenol as the sole carbon and energy source. Stock cultures of liquid grown phenol degrading cultures were harvested in late-log phase were frozen at -4°C in 20% (v/v) glycerol solution for preservation.

3.7.3 Growth Studies

3.7.3.1 Viable Cell Count

The growth of the bacterial consortium on phenol was studied as total viable count. The bacterial cells (10⁵ to 10⁶ cells in 5 mL) were added to the mineral salts medium containing phenol as sole carbon source. The conical flask was kept in orbital shaker at 150 rpm with an incubation temperature of 37°C. Samples were taken from the respective flasks every 24 h, and serially diluted for plating on nutrient agar. The viable bacterial
colonies on nutrient agar were counted using a colony counter (Schutt, Germany).

### 3.7.3.2 Total Protein

For analysis of total cell protein, samples were centrifuged at 12,000 rpm for 10 min and washed with fresh (substrate-free) mineral medium, then centrifuged and washed for second time. Each sample was then disrupted by sonication at 30% amplitude for a total of 3 minutes (1.5 min x 2) on an ice-water bath. Sample 0.5mL was added to 0.5mL of Coomassie Blue protein dye and the absorbance at 595nm was measured. Total protein concentration was determined using bovine serum albumin as standard (Bradford 1976).

### 3.8 ISOLATION OF PHENOL DEGRADING BACTERIAL STRAINS

Consortium from mineral salts medium with 50 g/L NaCl and 50 mg/L phenol after about 48 h of incubation, 1 mL of the culture was taken using sterilized pipette and serial dilution (10^{-3} to 10^{-10}) was carried out. The diluted suspension was transferred on to the agar plates by pour plate technique. The plates were incubated at 37°C for 48 h. After 48 h, discrete colonies developed on the surface of a nutrient agar plate, were picked with a sterile inoculation loop and aseptically transferred to separate nutrient agar slants containing the 50 mg/L of phenol. Each individual colony isolated from the consortium were grown on 100 mL of mineral salts medium in 250 mL Erlenmeyer flasks with optimum concentration of phenol (100 mg/L) and incubated for 48 h in rotary shaker at 150 rpm at incubated at room temperature. Morphology and motility of the strains grown in the exponential phase were examined as wet mounts in light microscope. The purity of the cultures was checked by plating them onto nutrient agar medium. The
consortium was maintained on mineral salts medium with phenol as the growth substrate and also the individual strains were maintained in mineral salts agar medium with phenol.

3.9 IDENTIFICATION OF THE PHENOL DEGRADING BACTERIAL STRAINS

The individual bacterial strains were separated from the consortium, which was used in the degradation of the phenolic compounds. The bacterial strains present in the consortium were examined using conventional biochemical tests (Cappuccino and Sherman 1999) initially. After which molecular techniques were used to identify the individual strains from the mixed DNA sample. Molecular identification of the bacterial strains was done by Cloning followed by 16S rRNA sequencing.

3.9.1. Biochemical characterisation

The bacterial strains present in the consortium were isolated and grown separately. Initially, Gram staining and motility test were performed after which biochemical characterization was done with KB003: Hi24 “Enterobacteriaceae Identification Kit”, (Himedia, India) to identify the phenotypic characters of the bacterial strains. The identification strip consists of 24 slots with different tests (Oxidase, ONPG, Lysine decarboxylase, Ornithine, Urease, Phenylalanine deamination, Nitrate reduction, H$_2$S Production, Citrate Utilization, Methyl red, Voges Proskauer, Indole production, and growth on Malonate, Esulin, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose and Lactose) to which 10 µL of 24 h old culture was added. After 24 h incubation at 37°C, the colour change observed was accounted for positive/negative result. Genus level identification of the unknown bacterial strains were accomplished by the using Bergey’s Manual of Systematic
Bacteriology (2005) to ascertain the existence of variable biochemical test results for each strain.

3.9.2 Genomic DNA Extraction and Amplification of 16s rDNA of the cultured isolates

Extraction of genomic DNA from stable enrichment cultures in MSM and the isolates was done by the method described by Yates et al (1997). The method was modified as follows: cell pellet of 2 ml from each enrichment culture and isolates was suspended in extraction buffer (100 mM Tris–HCl (pH 8.0), 100 mM Na₂EDTA (pH 8.0) Proteinase K (Invitrogen, USA) was added at the final concentration of 100 mg/mL and incubated at 55°C for 2 h with continuous shaking. NaCl (0.5 M) was added and incubated at 72°C for 30 min. Subsequently, DNA was extracted by phenol: chloroform: isoamyl alcohol (24:1). DNA was washed twice with 70 % ethanol and dissolved in Tris–EDTA buffer (pH 8.0) and analyzed by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining. DNA was also extracted from only autoclaved water to check for contamination from any of the reagents used for DNA isolation.

(i) Amplification, Cloning and Sequencing of 16S rDNA

The 16S rDNA of the enrichment cultures and isolates was amplified as described by Sambrook et al. (1989). The 1.5-kilobase partial sequence of the 16S rRNA gene was amplified from the pooled chromosomal DNA representing the bacterial strains using a polymerase chain reaction (PCR) and universal Eubacteria-specific primers 16F27 (5’-CCA GAGTTT GAT CMT GGC TCA G-3’) and 16R1525XP (5’-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3’).
The reaction mixture contained 1.5 mM MgCl$_2$, 0.2 M each dNTP, 25 pmoles each primer, 50 ng template DNA, and 2.5 U Taq DNA polymerase (Bangalore Genei, Bangalore, India) with a reaction buffer supplied by the manufacturer in a total volume of 100 ml. A hot start PCR was performed at 98°C 5 min before the addition of the Taq DNA polymerase. Thirty cycles of 60 sec at 94°C, 60 sec at 55°C, and 90 sec at 72°C, followed by a final extension of 12 min at 72°C was followed. A control PCR reaction containing all reagents was setup with autoclaved water instead of DNA to check for any nonspecific contamination.

The PCR products were purified using a QIA Quick gel extraction kit (Qiagen, USA) and then inserted into pGEM-T Easy vector (Promega, USA) following manufacturer’s protocols. The ligated vectors were inserted into *Escherichia coli* Bone shot^DH5a T1R MAX efficiency competent cells (Invitrogen, USA) by heat shock treatment. White colonies were picked and confirmed for inserts by alkaline-SDS rapid colony lysis method and PCR (Sambrook et al 1989). The PCR products were grouped according to the DNA patterns obtained by agarose gel electrophoresis after HaeIII digestion (Wright and Pimm 2003). Two representatives from each distinct pattern were selected for sequencing. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1625XP). An internal primer (16F536, 5’-GTG CCA GCA GCC GCG GTR ATA-3’) was also used in addition to the other primers. The sequencing of the 16S rRNA gene insert from the clones was done using the 16F27N primer. The 5’-terminus, a 500-nucleotide region of the 16S rRNA gene, contains variable regions (positions approximately 100-200) that not only discriminate between closely related species, but in
most cases will also underestimate the degree of sequence similarity of nearly complete sequences by 1–12% for strains belonging to different phyla.

Sequencing was performed on 3730 DNA analyzer (Applied Biosystems, USA) using ABI Big-Dye Version 3.1 sequencing kit, as per manufacturer’s instructions. In the case of isolates, 30 colonies were selected randomly for each isolate and checked for inserts. Five positive clones from each isolate were sequenced. The partial 16S rDNA sequences of the enrichment culture clones and isolates were deposited to Gen Bank and the accession numbers are EU780459, EU780460, EU780461, EU780462, EU780463, and EU780464.

(ii) **Phylogenetic Analysis**

The nucleotide sequences obtained from the ABI DNA analyzer was studied using BLAST software available in National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). After editing the sequence, was analysed with BLAST software to identify the specific type of bacterium corresponding to the nucleotide sequence. An equal portion (approximately 500 base pairs) of the 16S rRNA gene (E. coli positions 67 to 572, Accession number J01859), was used for sequence analysis at the Ribosomal Database Project (RDP II; Michigan State University, East Lansing, MI) and the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST). The similarity matrix was prepared using the similarity matrix calculator at the RDP II site. The presence of chimeric sequences was checked with the RDP Chimera Check program and by comparing independently the alignments at the beginning of each sequence and at the end of each sequence and the alignments of the entire sequence. The alignment of all the 16S rRNA gene sequences was done using CLUSTAL W program at the European Bioinformatics server (European Bioinformatics Institute, Wellcome Trust
Genome Campus, Hinxton, Cambridge, United Kingdom) (http://www.ebi.ac.uk/clustalw) to determine similarity value and distance matrix. Further, the phylogenetic tree was constructed using 500 base pair aligned sequences. Phylogenetic analyses were conducted in MEGA4 (Tamura et al 2007).

3.10 BIODEGRADATION PHENOLIC COMPOUNDS

Bacterial consortium was inoculated at a concentration of $8 \times 10^5$ cfu/mL in mineral salts medium, with selected phenolic compounds as sole carbon source. The different experiments conducted for degradation study are given in Table 3.2.

Table 3.2 Experimental set up for Phenolic compounds degradation

<table>
<thead>
<tr>
<th>S.No</th>
<th>Combination</th>
<th>Experimental setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium + Phenolic compound</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>Medium + Bacterial Strain/ Consortium</td>
<td>Control</td>
</tr>
<tr>
<td>3</td>
<td>Medium + Phenolic compound + Bacterial strain/consortium</td>
<td>Sample</td>
</tr>
</tbody>
</table>

All the combinations were prepared in duplicates and kept for incubation at 37°C in an orbital shaker at 150 rpm. The contents of the whole flask were extracted at 24 h time intervals for 5 days. The samples were extracted twice with dichloromethane (100 mL) after acidification to pH 2.5 with 1N HCl. The residual phenolic compound left over in the conical flask was also extracted with dichloromethane. The extract was filtered through anhydrous sodium sulphate in order to remove the water content. After filtration, the extract was condensed to a final volume of 1mL using rotary evaporator (Buchi rotavapour R-124, Switzerland) for gas chromatographical analysis of the phenolic compounds. The condensed solvent extract was
filtered through 0.2 mm syringe filter and analysed in Gas Chromatography (GC).

3.10.1 Effect of Salt Concentration on the Degradation of Phenolic compounds

To study the effect of salt on the degradation of phenol, batch studies were conducted at different concentrations of NaCl (10g/L, 30g/L, 50g/L, 70g/L, 100g/L, and 150g/L) in order to find out optimum NaCl concentration at which removal efficiency was maximum. Degradation of phenol was analyzed in GC, and based on the maximum removal efficiency, the NaCl concentration was chosen as the optimum concentration for further degradation of phenolic compounds.

3.10.2 Effect of pH on the Degradation of Phenolic compounds

To study the effect of pH, a range of pH was selected from (5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) the consortium was inoculated in the flasks containing 50mg/L of phenol with 50g/L of NaCl. The flasks were incubated at 37°C in an orbital shaker at 150 rpm. The removal efficiency of phenol was noted by withdrawing the sample at every 24 h. The pH, which gave the maximum percentage of phenol degradation, was considered as the optimum pH.

3.10.3 Biodegradation of Different Concentrations of Phenolic Compounds at Optimum salinity

To check the ability of the consortium to grow on different phenolic substrates, the mineral salts medium was inoculated individually with different phenolic substrates. Each individual substrate was used at different concentrations as mentioned in Table 3.3.
Table 3.3 Concentrations of Phenolic compounds used in this study

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Names of Phenolic compounds</th>
<th>Concentration of Phenolic compounds (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenol</td>
<td>50,100,150,200,250,300</td>
</tr>
<tr>
<td>2.</td>
<td>o-Cresol</td>
<td>50,75,100</td>
</tr>
<tr>
<td>3.</td>
<td>m-Cresol</td>
<td>50,75,100</td>
</tr>
<tr>
<td>4.</td>
<td>p-Cresol</td>
<td>50,75,100</td>
</tr>
<tr>
<td>5.</td>
<td>Dual substrates (Phenol, o-Cresol)</td>
<td>100 and 50</td>
</tr>
<tr>
<td>6.</td>
<td>4-Chlorophenol (4-CP)</td>
<td>25,50,75,100</td>
</tr>
<tr>
<td>7.</td>
<td>2,4-Dichlorophenol (2,4- DCP)</td>
<td>25,50,75,100</td>
</tr>
<tr>
<td>8.</td>
<td>2,4,6-Trichlorophenol(2,4,6-TCP)</td>
<td>25,50,75,100</td>
</tr>
</tbody>
</table>

3.10.4 Effect of Nitrogen sources on the Degradation of phenolic Compounds

To study the effect of nitrogen source the mineral salts medium with 50g/L of NaCl containing 0.01 % yeast extract was replaced with different nitrogen sources such as tryptone (0.01 %) and urea (0.01 %) incubated for 37°C in an orbital shaker at 150 rpm and analyzed for phenolic compounds removal efficiency by GC.

3.10.5 Effect of Additional Salts on the Degradation of Phenolic Compounds

Effects of additional salts on the biodegradation of phenolic compounds were determined by addition of the 5 % (v/v) individual salts such as KCl, Na₂SO₄, K₂SO₄ and NaNO₃ to the MSM and incubated for 37°C in an orbital shaker at 150 rpm and analyzed for phenolic compounds degradation efficiency by GC.
3.11 ENZYME ASSAY

3.11.1 Preparation of Cell-free Extract

The bacterial consortium grown individually on phenol (100 mg/L), and 4-chlorophenol (25 mg/L) at an optimum salinity of 5% was harvested at the exponential phase by centrifugation and washed 0.033 M Tris–HCl buffer (pH 7.6) to remove the salts and resuspended in the same buffer. Cells were disrupted by using the tip sonicator Bandelin Sonopuls GM 200. Polyvinylpolyprrolidone (PVP) was added to the suspension to remove the phenolics. The samples were centrifuged at 15,000 rpm for 30 min at 4°C and the pellet was discarded. The clear supernatant solution was used as crude enzyme assay. The cell free extract was kept in ice and assayed for dioxygenase activity.

3.11.2 Dioxygenase Activity

Catechol 1,2 dioxygenase (Type I) activity on the degradation of phenol was measured by following the formation of cis,cis- muconic acid, the ortho-cleavage product of catechol. The following reagents were added in the quartz cuvette 2mL of 50mM Tris HCl buffer (pH 8.0); 0.7 mL distilled water 0.1 mL, 100 mM 2-mercaptoethanol; 0.1 mL cell-free extract. The contents of the cuvette were mixed by inversion and 0.1 mL catechol (5µM) was then added and the contents were mixed again. Catechol 1,2 dioxygenase was assayed following the formation of cis,cis muconic acid. The increase in the absorbance at 260 nm and decrease in the absorbance at 278 nm over a period of 5 mins were followed in a Speckol spectrophotometer (Ngai et al 1990).

Catechol 1,2-dioxygenase (Type II) activity on the degradation of 4-chlorophenol was measured by following the formation of 2-chloromuconic acid, the ortho- cleavage product of 3-chlorocatechol. The procedure used was as same as Type I activity, with 3-chlorocatechol (5µM) being used in the place of catechol (5µM) (Farrell and Quilty 1999).
Catechol 2,3-dioxygenase activity on the degradation of phenol and 4-chlorophenol, was measured by following the formation of 2-hydroxymuconic semialdehyde, the meta-cleavage product of catechol, or the formation of 5-chloro-2-hydroxymuconic semialdehyde, the meta-cleavage product of 4-chlorocatechol. The following reagents were added to a cuvette: 2 ml 50 mM Tris-HCl buffer (pH 7.5); 0.6 ml distilled water; 0.2 ml cell free extract. The contents were mixed by inversion and 0.2 ml catechol or 4-chlorocatechol (50 mM) was added and mixed with the contents. The production of 2-hydroxymuconic semialdehyde was followed by increase in absorbance at 375 nm over a period of 5 mins, while the production of 5-chloro-2-hydroxymuconic semialdehyde was followed by the increase in absorbance at 380 nm over the same period (Farrell and Quilty 1999). Protein estimation was measured by bradford's method by using bovine serum albumin as a standard (Bradford 1976). Specific enzyme activities are reported as µmol/min/mg protein. All assays were performed in duplicates.

3.12 BIOREACTOR STUDIES WITH PHENOL CONTAINING SALINE WASTEWATER

A bioreactor of 4L capacity was used for laboratory study in the treatment of phenol containing saline wastewater. The phenol containing saline wastewater was collected from a pharmaceutical company near Ennore. The sample was extracted with dichloromethane and analysed by GC-MS for identifying the phenolic compounds in the phenol contaminated saline wastewater. The consortium was acclimatized in the phenol containing wastewater for 5 days for scaling up the volume of the consortium. The bioreactor was filled with 3 L of the phenol contaminated saline wastewater to which 250 mL of bacterial consortium (10^5 to 10^6CFU/mL) was added. Temperature was maintained at 37ºC and continuously aerated with constant agitation using stirrer at 300 rpm. The samples from the reactor were analysed for chemical parameters such as pH, COD, BOD, MLSS, MLVSS, Total
phenols, Total Kjeldhal Nitrogen and Phosphate (APHA 2005) every 24 h. Phenolic compounds present in the raw saline wastewater and the degraded products were analysed by GC-MS.

3.12.1 Biomass Estimation

For the determination of biomass concentration as dry weight, 10 ml aliquots were centrifuged for 15 min at 15000 rpm (Biofuge Stratos, Heraeus instruments, Kendro, Hanau, Germany) in eppendorf tubes. The pellets were dried at 60°C for 48 h and cooled in a desiccator at room temperature and weighed. The difference in weight between the empty tubes and the tubes with dried pellet gives the dry cell weight of the biomass expressed in mg/L.

3.13 INSTRUMENTATION

3.13.1 Gas Chromatographic (GC) Analysis

The GC analysis was performed with Chemito 1000 unit equipped with a (Chemito GC Model No 1000) equipped with FID detector and capillary column (Varian Chromopak capillary column CP SIL 8 CB, 30m X 0.32 mm. Nitrogen was used as a carrier gas, injector temperature was 220°C, detector temperature was 250°C and the oven temperature of the column was maintained at 150°C, connected to WINCHROME software, which was used to process the data. A standard solution of different phenols were prepared and used as reference for the test samples. The samples were injected one by one and the extent of degradation of phenols was found out depending on the percent peak area and retention time. Quantification was done using standards for each phenolic compound. The lowest detection range using the phenol specific column was 10 – 20 ng/mL.
3.13.2 Gas Chromatography-Mass Spectroscopy (GC-MS)

A GC-MS analysis was performed with GC-MS-QP2010 [SHIMADZU] with an inert mass selective detector and a computer workstation was used for the Phenolic compounds analysis. The samples were silylated before analysis. The GC–MS was equipped with: an Agilent DB-5 capillary column (30m x 0.25mm id x 0.25 µm); with an injection volume of 1 µL, split ratio of 20 injection at 280°C and an ion source temperature at 200°C. Oven operating temperature was 80°C with the holding time of 1 min, 300 ºC for 2 mins with the total time of 41.67 mts. The masses of primary and secondary phenolic compound ions were determined by using the scan mode with impact ionization (70 eV, 200°C) for pure phenolic compounds standards (Merck). Qualitative analysis of Phenols was performed by using the selected ion monitoring (SIM) mode. Fragmented products were identified using computer station library search. Retention time of the fragmented products are further compared and confirmed by analyzing authentic standards. Helium was used as the carrier gas.

3.13.3 Scanning Electron Microscopy (SEM)

The sample preparation for Scanning Electron Microscopy (SEM) was carried out according to the method of Prior and Perkins (1974). The isolated bacterial strains were grown individually on MSM for 24 h. The bacterial strains in the mineral salts medium were centrifuged at 8000 × g for 10 min and the pellets were immediately re-suspended in 2% Glutaraldehyde with 0.05 M phosphate buffer and 4% sucrose (pH 7.3). Fixation was obtained overnight at 4°C. After 24 hours the pellets were centrifuged at 8000 x g for 10 min, washed 4 times with distilled water, placed on aluminum foil. The samples were then dehydrated with series of gradient ethanol (i.e 10%, 20%, 30% till 90%) air dried and finally the dried flakes were coated with platinum and examined under SEM (JEOL JSM-6360).