Chapter 5
5. Materials and Methods

5.1 Development of anoxic phenolytic cultures

Anoxic mixed phenolytic enriched cultures were developed from the following sludge as well as samples were collected from contaminated sites.

1. (AS) Activated Sludge from the full scale treatment plant treating Kanpur City domestic wastewater.
2. (S) Soil from Kanpur City solid waste dumpsite.
3. (SCO) Soil from the vicinity where wastewater from a steel plant is discharged, Durgapur.
4. (PCO) Soil from the vicinity where coke oven wastewater of a Thermal Power plant is discharged, Durgapur.
5. (COW) Sludge from the coke oven Phenolic Wastewater treatment site, steel industry at Durgapur.
6. (ANS) Anaerobic sludge from the full scale treatment plant treating Kanpur City domestic wastewater.
7. (PWI) Sediments from a pond receiving Phenolic Wastewater from plywood industries, Unnao (U.P)

2.5-L aspirator bottles with 2.0-L liquid volume were used as reactors for developing cultures. 20 g of either sludge or soil (wet weight) was used
as the seed inoculum. Feed solution consisted of organic carbon source, KNO₃ and Nutrient medium (10 ml) per liter of tap water. Composition nutrient medium was as follows:

KH₂PO₄, 4 g; MgSO₄, 0.1 g; Yeast extract, 1.0 g; Trace metal sol. 2 ml per litre of distilled water. Trace metal solution consisted of:

FeSO₄.7H₂O, 1.36 g; Na₂MoO₄.2H₂O, 0.24 g; CuSO₄.5H₂O, 0.25 g; ZnSO₄.7H₂O, 0.58 g; NiSO₄.6H₂O, 0.11g; MnSO₄.H₂O, 1.01 g; H₂SO₄ 1 ml per litre of distilled water. Initially 250 mg.L⁻¹ acetate and 100 mg.L⁻¹ phenol served as organic carbon source. Enough KNO₃ was added to the reactor so as to maintain organic carbon to nitrate N ratio in the range of 0.85-1. Once the phenolytic activity was established, phenol concentration in the feed was increased gradually, while sodium acetate levels were decreased. All reactors were maintained at a temperature range of 30 - 35 °C and operated in once fed mode. Daily maintenance of the reactors included wasting of required volume of reactor contents and replacing the same volume with feed solution. Initially 8 day hydraulic retention time (HRT) was maintained. 250ml of the reactor content was replaced by the fresh feed solution everyday. After two or three weeks, HRT was gradually decreased to 1.3 day, which was then maintained constantly through out the study.
5.2 Studies with suspended enriched cultures

5.2.1 Development of enriched culture

Suspended mixed culture was maintained in a 500ml bottle fitted with butyl rubber stopper. Initially 50ml culture from 2-L reactor were transferred to 450 ml basal medium. Basal medium used for the maintenance of the enriched culture consisted of \( \text{KH}_2\text{PO}_4 \cdot 4 \text{ g} \); \( \text{K}_2\text{HPO}_4 \cdot 6 \text{ g} \); \( \text{KNO}_3 \cdot 1.1 \text{ g} \); \( \text{NaHCO}_3 \cdot 0.84 \text{ g} \); \( \text{MgSO}_4 \cdot 0.25 \text{ g} \); \( \text{CaCl}_2 \), 0.035 g; Yeast extract 0.1 g; trace metal solution.; 1 ml per litre of distilled water.

Phenol concentration was maintained at 200 mg.L\(^{-1}\). The bottle, was kept in a incubator at 35 °c under static conditions. When phenol was completely depleted, accompanied by nitrate removal, 50 ml of this culture was again transferred to 450 ml of basal medium containing 200 mg.L\(^{-1}\) of phenol. For any given experiment, in which different test conditions were used, inoculum was from the same maintenance culture bottle.

5.2.2 Comparison of the medium for enriched phenolytic cultures

Three media were compared for the phenol degradation. Compositions of these media are

Medium 1 - This is the same as the maintenance medium except yeast extract used was 10 mg.L\(^{-1}\).
Medium 2 - Na$_2$HPO$_4$·2H$_2$O, 3.10 g; KH$_2$PO$_4$, 1.60 g; NaHCO$_3$, 0.84 g; NH$_4$Cl, 0.50 g; MgSO$_4$·7H$_2$O, 0.10 g; CaCl$_2$·2H$_2$O, 0.02 g; Yeast extract, 10 mg; Trace metal solution 1.0 ml per litre.

Medium 3 - NaHCO$_3$, 0.15 g; KH$_2$PO$_4$, 0.025 g; MgSO$_4$, 0.005 g; CaCl$_2$, 0.005 g; FeCl$_3$, 0.0025 g; Yeast extract, 10 mg per litre.

Three media were compared for the removal of 100 mg L$^{-1}$ of phenol. KNO$_3$ was added to each of these media in stoichiometric quantities. 95 ml of the medium was taken in 125 ml of serum bottles, flushed with N$_2$ and fitted butyl rubber cork. 5 ml of the inoculum from the maintenance culture was added to each bottle through the septum. Aliquots were withdrawn for the determination of phenol & nitrate at different time intervals. Based on the phenol removal kinetics, medium 1 was chosen for all further experiments.

5.2.3 Phenol degradation kinetics

This study was carried out using 125ml serum bottles. After flushing 95 ml medium with Nitrogen, required volume of phenol so as to have initial phenol concentration in the range of 100-300 mg L$^{-1}$ and 5ml inoculum from maintenance culture were added. Phenol was determined in an aliquot taken out at different time intervals. This study carried out with enriched cultures, (COW), (PCO), and (AS), so as to compare the mixed culture phenolic activity. Since (COW) gave marginally better
degradation rate as compared to (PCO) and (AS), hence this culture was used for all further studies.

5.2.4 Determination of Biokinetic Constant

Biokinetic constants of phenolytic anoxic culture were determined using Batch as well as Semi-continuos modes

5.2.4.1 Batch Mode: Batch growth studies with phenol were conducted in 125ml serum bottles containing 90ml medium. Each growth bottle contained a different initial phenol concentration in the range of 20-250 mg.L⁻¹. KNO₃ was added to each flask proportionately so as to maintain the organic carbon / NO₃-N ratio of 1. 5ml inoculum was added to each bottle from the same maintenance bottle. Optical density was measured frequently at 550nm to assess the biomass growth. In order to eliminate the effect of flocculation and deflocculation of microbial cells (since incubation was carried out under static conditions), the samples were mixed for a minute using a laboratory vortex mixer before measuring the absorbance. Phenol concentration was monitored by colorimetric method after removing the cells by centrifugation. Atleast 8 - 10 data points were collected during each experiment.

5.2.4.2 Semi-continuous Mode: Two 500 ml reactors were maintained at 3.0 day HRT at 35⁰C ± 1°C. 167 ml of mixed culture medium was removed from the reactor every 24hr and was replaced with fresh medium containing 200 mg.L⁻¹ phenol. After atleast five turnovers of
HRT, phenol depletion with time was monitored within one waste feed cycle at two initial phenol concentration i.e 100 and 200 mg.L$^{-1}$.

### 5.3 Granular sludge development

An experimental conditions described in 5.1 was used for granular sludge development.

*Sludge volume index (SVI):* SVI is determined by measuring the sludge volume after it has settled for 30 minute and is given by following formula:

\[
    \text{SVI} = \frac{V \times 1000}{\text{MLSS}}
\]

- $V =$ volume of settled sludge (ml.L$^{-1}$)
- MLSS = Mixed liquor suspended solids

### 5.4 Batch studies with anoxic granular sludge

Batch studies with anoxic granular sludge were carried out in 125 ml serum bottles containing 100 ml medium. Sludge concentration was generally maintained at 0.328g/L (dry weight). After flushing the medium and the head space with nitrogen, bottles were sealed with butyl rubber stoppers. Required volume of phenolic substrates was added from the respective stock solution. Bottles were kept in a rotary shaker (Remi Model) at 100 rpm. Appropriate controls like bottles with phenol substrate in the absence of biomass or in the presence of heat killed biomass were always included with each run.
5.4.1 Comparison of phenolytic activity of granular sludge developed from different source inoculum

A known quantity of granular sludge (0.328g/L) was taken in 125ml serum bottles that contained 100ml medium. Medium was flushed with nitrogen to establish anoxic conditions. Bottles were incubated at 35°C and constantly shaken at 100 rpm in rotary shaker (Remi, Model) to keep the granules in suspension. Phenol concentration range of 100-300 mg.L⁻¹ used in this study. Phenol removal kinetics was determined by monitoring phenol and nitrate periodically.

5.4.2 Removal of phenolics in mono-substrate and binary-substrate matrix by the granular sludge in batch mode

Experiments were carried out in 125ml serum bottles with 100ml liquid volume containing nutrient and required quantities of KNO₃. Phenol adapted granules from 2.5-L once fed semi-continuous reactors were added to these bottles at a concentration of 0.328 g/L (dry weight). Required quantities of specific phenols were added so as to maintain the concentration at 100 mg.L⁻¹ from the respective stock solution, through the septum. Bottles were kept in the shaker at 35°C. One bottle with phenol as the organic carbon was included for comparison purpose.
Studies with binary substrate matrix were carried out using phenol (100 mg.L\(^{-1}\)) as one of the substrates in combination with catechol, \(p\)-Hydroxy benzoic acid and cresols. Aliquots were removed from the bottle at periodic intervals and analysed for residual phenolic compound either by UV, Colorimetric or HPLC methods.

5.4.3 Phenol removal rates in the presence of easily assimilable organics

100 ml of synthetic wastewater containing phenol and glucose (or acetate), each at a concentration of 100 mg.L\(^{-1}\) was taken in 125 ml serum bottles. 5 ml nutrient solution and enough KNO\(_3\) required for the removal of phenol and glucose or (acetate) was provided. Sufficient granular biomass to maintain MLSS concentration of 0.328 g/L (dry weight) was added to each bottle. Bottles were then flushed with nitrogen and sealed. Phenol removal was periodically monitored whereas initial and final chemical oxygen demand (COD) and nitrate was estimated. When phenol was completely depleted in the first cycle, supernatant was replaced with a fresh medium containing phenol and glucose (or acetate) and a second cycle of phenol depletion in the presence of glucose/acetate was carried out as described above. This procedure was repeated three times.
5.4.4 Anoxic granules exposed to aerobic conditions

After one cycle of phenol (100 mg.L\(^{-1}\)) depletion under denitrifying conditions as described in 4.4, the granules were exposed to aerobic conditions. To achieve this, the supernatant was replaced by fresh minimal medium having phenol (100 mg.L\(^{-1}\)) but lacking in nitrate. Instead ammonium chloride was provided. The bottles were covered with punched aluminium foil and shaken at 100 rpm at 35\(^{\circ}\)C. This exposure of granules to aerobic conditions was repeated for 3 consecutive cycles. After the third cycle, the granules were once again exposed to anoxic conditions. Phenol removal was monitored in each of these cycles periodically.

5.5 Studies on anoxic phenol removal in sequential batch reactors

5.5.1 Experimental setup

The reactor consisted of cylindrical perspex column (dia 6.5 cm, length 60 cm) (Fig 5.1). Total liquid volume in the reactor was 1650 ml. Granular sludge developed from (COW) and (AS) were used in this study. Waste entered horizontally at the bottom through the sludge bed. Biogas exited through the port at the top. The reactor was maintained at ambient temperature which ranged from 30-35 \(^{\circ}\)C. A synthetic wastewater containing phenol and KNO\(_3\) in the ratio of 1:0.85 with
Fig 5.1 Schematic diagram of the experimental setup of sequential batch reactor

1 Feed Container
2 Feed Pump
3 SB Reactor
4 Recycle Pump
5 Water Seal
respect to organic carbon and NO$_3$-N; phosphate and other nutrients were prepared in tap water freshly for each run. The reactor was filled with wastewater during discrete period of time and was then operated in a batch treatment mode. No mixing was given in the filling time in the present study. Liquid recirculation at 250 ml/hr was given during the batch react period. In the present study a mesh was provided at few cm below recirculation port to avoid the floating granules entering the recycle port and getting into the peristaltic pump. After the reaction period, waste sludge mixture, was allowed to settle for 2 hours and then the supernatant was withdrawn. Volume withdrawn after react and settle period was 1.15 L. Under each experimental condition, SBR was operated for at least 15-20 cycles. Ambient temperature during this study was in the range of 30-35 °C.

5.5.2 Experimental protocol

<table>
<thead>
<tr>
<th>Table 5.1 Operating Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle length (hr)</td>
</tr>
<tr>
<td>Liquid Volume in the reactor (L)</td>
</tr>
<tr>
<td>Liquid Volume withdrawn/ Cycle (L)</td>
</tr>
<tr>
<td>Organic Loading (gCOD/L/d)</td>
</tr>
</tbody>
</table>

* Depending upon the initial phenol concentration and cycle length
Table 5.2 Experimental conditions

<table>
<thead>
<tr>
<th>Operating Sequence (hr cycle)</th>
<th>Fill time (hr)</th>
<th>Recycle time (hr)</th>
<th>Settle time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>9/6</td>
<td>13/16</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>6/3</td>
<td>4/7</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

5.6 Microbial characterization of anoxic granules

Microbial characterization was carried on the anoxic granules using Gram’s stain.

Reagents: (a) Crystal violet: Mixture of reagent A + B

Reagent A
- Crystal Violet, 2 g
- Ethyl Alcohol, 20 ml

Reagent B
- Ammonium oxalate, 0.8 g
- Distill water, 80 ml

(b) Ingol’s Iodine:

Procedure: Anoxic granules were taken from the 2-L reactor and were macerated in a sterile waster. Inoculum from these granules was smeared on the slide and was heat dried to fix the smear onto the slide.
Crystal violet, iodine, alcohol and Safranin was added for one minute each and then wiped off with water. This stained smear was seen under the compound microscope.

5.7 Analytical Methods

Liquid samples were filtered through 0.45 µm filter for the analysis of soluble components. Filtration of the sample was intended to remove possible interference from suspended particles.

5.7.1 Estimation of phenolic compounds

The following methods were employed to estimate the phenolic compounds:

(a) 4-aminoantipyrene method
(b) Folin-Lowry method
(c) UV spectrophotometry
(d) HPLC analysis

5.7.1 (a) 4-aminoantipyrene method (Standard Methods)

Phenolic compounds, phenol and o-cresol were estimated colorimetrically using 4-aminoantipyrine reagent as per the procedure described in Standard Methods.

Principle: Phenolic compounds (exception being m- and p- substituted phenols, e.g. m- and p-cresols that are less sensitive to 4-
aminoantipyrene,) react with 4-aminoantipyrene at pH 7.9 ± 0.1 in the presence of potassium ferricyanide to form a coloured antipyrene dye whose absorbance is measured at 510 nm. The minimum detectable quantity is 10 µg phenol when a 5-cm cell and 100 ml sample are used.

Reagents: (a) 12.5 g Ammonium chloride in 125 ml Ammonia made upto 250 ml (b) 2% 4-aminoantipyrine and (c) 8% potassium ferricyanide.

Procedure: To 5 ml samples containing phenol concentration in the range of 100 mg.L⁻¹, 0.5ml ammonia-phosphate buffer, 0.1ml aminoantipyrine solution and 0.1ml ferricyanide were added in succession, with thorough mixing after each addition. Absorbance of red colored complex was measured against reagent blank at 510 nm after 5 minutes (Systronics Model 106, India). Calibration graph was prepared with phenol in the concentration range of 0-100 mg.L⁻¹ using the same procedure.

5.7.1 (b) Folin-Lowry method (Plummer, DT)

The phenolics (catechol, resorcinol, m- and p-cresols) were estimated by the Folin-Lowry method Principle: Phenols react with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is
due to the reaction of the alkaline copper with the phenol and the reduction of phosphomolybdate.

Reagents: (a) 2% Na$_2$CO$_3$ solution in 0.1 N NaOH
(b) 0.5% CuSO$_4$
(c) 1% Na, K tartarate
(d) Folin-Ciocalteau reagent (commercially available, E. Merck) – diluted 1:1

Procedure: To 1 ml or an appropriately diluted 1 ml sample, 5 ml of alkaline solution (freshly prepared by mixing 1 ml, each of CuSO$_4$ and Na, K tartarate and 10 ml of Na$_2$CO$_3$ solution) was added. The sample was mixed thoroughly and allowed to stand at room temperature for 10 min. Folin-Ciocalteau reagent (0.5 ml) was added later with immediate mixing. The absorbance was read after 20 min against appropriate blank at 660 nm (Systronics Model 106, India). A standard curve was prepared by plotting absorbance of standards of the phenolics (treated under similar conditions) against its concentration (0–100 mg.L$^{-1}$). Sample concentration was obtained from the standard curve.

5.7.1 (c) U.V. Spectrophotometry

Principle: A U.V. spectrophotometer measures how much of light is absorbed while passing through a sample. The spectrophotometer can be set to a particular wavelength of light that is chosen on the basis of the absorbance spectrum of the compound.
**Procedure:** The concentrations of the phenolic compounds, like p-hydroxybenzoic acid, benzoic acid and salicylic acid in the growth media were determined by recording the UV spectra of appropriately diluted samples of culture supernatants (Shimadzu UV-Vis Recording Spectrophotometer Model UV-160A). The concentration of a phenolic compound was calculated from the extinction value (e.g., Molar extinction coefficient of p-hydroxybenzoic acid with 1 cm light path is 10,419) at their absorption maximum of the respective compound.

5.7.1 (d) High Performance Liquid Chromatography (HPLC)

**Principle:** In HPLC, eluent from the solvent reservoir is filtered, pressurized and pumped through the chromatographic column. A mixture of solutes injected at the top of the column is separated into components on travelling down the column and the individual solutes are monitored by the detector and recorded as peak on the chart recorder. The main components of a high performance liquid chromatography are a high-pressure pump, a column/injector system and a detector. In addition, components such as solvent reservoirs, in line filters, pressure gauges, recorders, integrators and minor components may be required.

*Reversed Phase column:* In the present study, reverse phase column (C18) was used. Reversed phase columns address the widest range of applications of any HPLC column type. They are prepared by binding alkysilane reagent (octadecyl) to the base 5µ spherical silica.
Consequently, when an unknown mixture is to be analysed, a reversed phase column is often tried first. Retention in reversed phase liquid chromatography depends on the relative hydrophobicity of the sample compounds. As expected, on a non-polar surface, the less polar the solute, the longer the retention. Polar functional groups in a molecule tend to decrease the attraction on the reversed phase packing.

The analytical column used in the present study had the following specifications: 4.0 x 250 mm, Particles, 5 µm and packing of Spherisorb ODS 2.

*Solvent System:* A solvent mixture having Methanol: H₂O: Acetic acid in the ratio of 60:38:2 was used for the separation of phenolic components.

The flow rate of the solvent was always maintained at 0.5 ml/min. Phenolic compounds were estimated at 274 nm. Retention time of few phenolics under given experimental conditions is as follows:

*p*-hydroxybenzoic acid 4.54 min, Phenol 5.15 min, and Cresols 6.06 min.

### 5.7.2 Nitrate (Standard methods)

Measurement of UV absorption at 220 nm enables the rapid determination of nitrate (Standard Methods).

*Principle:* The nitrate calibration curve follows Beer's law upto 11 mg NO₃-N/L. As dissolved organic matter may also absorb at 220 nm and nitrate does not absorb at 275 nm, a second measurement was made at 275 nm. To obtain the total nitrate in the sample, two times the
absorbance at 275 nm was subtracted from the reading at 220 nm. The extent of this empirical correction is related to the nature of concentration of organic matter and may vary from one sample to another and maybe useful for measuring samples with a constant type of organic matter. Nitrite interference was compensated for by an independent determination of its concentration by the diazo method, and preparation of correction curve and its deduction from total nitrate to arrive at the nitrate concentration in the sample. Acidification with 0.5 N hydrochloric acid is designed to prevent interference from hydroxide or carbonate concentration up to 1000 mg.L⁻¹ CaCO₃. Chloride has no effect on the determination.

Reagents: (a) 0.5 N HCl solution. Redistilled water was used for the preparation of all solutions and dilutions.

Stock nitrate solution: KNO₃ was dried (105 °C/24 h) and 0.7218 g dissolved in water and diluted to 1000 ml: 1 ml = 100 µg NO₃⁻N. It was preserved with 2 ml CHCl₃.L⁻¹.

Intermediate nitrate solution: 100 ml stock nitrate solution was diluted to 1000 ml with water: 1 ml = 10 µg NO₃⁻N and preserved with 2 ml CHCl₃.L⁻¹.

Procedure: A series of 10 ml nitrate standards containing 0 – 70 µg NO₃⁻N.L⁻¹ was prepared by diluting appropriate volumes of intermediate nitrate solution. The standards as well as required aliquot of sample diluted to 10 ml were treated with 0.2 ml 0.5N HCl and the absorbance
was read against distilled water set at zero absorbance or 100% transmittance (Shimadzu UV-Vis Recording Spectrophotometer Model UV-160A).

5.7.3 Nitrite (Standard methods)

Principle: Nitrite is determined through the formation of reddish purple azo dye produced at pH 2.0 to 2.5 by coupling of diazotized sulfanilic acid with N-ethylenediamine dihydrochloride (NEDA). The diazotization method is suitable for the determination of nitrite nitrogen in the range of 1-25 µg.L⁻¹ N.

Reagents: (a) Sulfanilamide reagent (5 g sulfanilamide in 50 ml concentrated HCl and 450 ml distilled water)

(b) N-(1-napthyl) ethylenediamine dihydrochloride solution (500 mg NEDA in 500 ml distilled water)

Stock nitrite solution: 1.232 g NaN0₂ was dissolved in nitrite free water and diluted to 1000 ml (0.05 M): 1 ml = 250 µg NO₂-N and standardized against standard KMNO₄. It was preserved with 1 ml chloroform.

Intermediate nitrite solution: Dilute 50 ml stock nitrite solution to 250 ml nitrite free distilled water: 1 ml = 50 µg NO₂⁻ N.

Standard nitrite solution: Dilute 10 ml intermediate nitrite solution to 1000 ml with nitrite free water; 1 ml = 0.5 µg NO₂⁻ N. Both intermediate and standard nitrite solutions were prepared daily.
Procedure: To 10ml sample or to a portion diluted to 10ml and neutralized to pH =7.0, 0.2ml sulfanilamide solution was added. The reagent was allowed to react for 2 min followed by addition of 0.2ml NEDA. The sample was mixed immediately and the absorbance was measured at 545 nm after 30 min (Systronics Model 106, India). A calibration curve was prepared by plotting absorbance of standards against NO₂⁻ N concentration, (0-0.4 mg.L⁻¹) and the sample was concentration obtained by referring to the calibration curve.

5.7.4 Ammonia (Standard methods)

Ammonia was measured colorimetrically by Nesslerization

Principle: Ammonia was measured colorimetrically by Nesslerization. The nessler method is sensitive to 20 µg NH₃-N.L⁻¹ under optimum conditions and maybe used up to 5 mg NH₃-N.L⁻¹. Turbidity, color and substances precipitated by hydroxyl ion such as magnesium and calcium, interfere and were removed by precipitation with 10 % zinc sulphate. Rochelle salt solution was added to inhibit precipitation of residual calcium and magnesium ions in the presence of nessler's reagent. The yellow colour characteristic of low NH₃-N concentration (0.4 – 5 mg.L⁻¹) was measured at 400 (Systronics Model 106, India).

Reagents: (a) Nessler’s reagent (commercial reagent)
(b) ZnSO₄ (10%)
(c) Rochelle salt solution (KNaC₄H₄O₆.4H₂O)
Stock ammonium solution: Anhydrous NH₄Cl was dried (100 °C) and 3.819 g was dissolved in water and diluted to 1000 ml; 1 ml = 1 mg NH₃-N = 1.22 mg NH₃.

Standard ammonium solution: Stock ammonium solution, 10 ml, was diluted to 1000 ml with water; 1 ml = 10 µg NH₃-N = 12.2 µg NH₃.

Procedure: Sample or a portion was diluted to 10 ml with water and to this, 0.1 ml of 10 % ZnSO₄ was added and mixed thoroughly. Add 0.4 ml 6 N NaOH solution to obtain a pH of 10.5 whereupon a floc was formed. The supernatant was centrifuged and 5 ml taken and 1-2 drops of Rochelle salt solution added and mixed. To this, 0.1ml nessler reagent added and the samples were mixed thoroughly. After 10 min, the absorbance was measured at 440 nm against a reagent blank. Calibration curve was prepared using NH₄Cl as standard in the concentration range, 0-5 mg.L⁻¹ under conditions identical with those adopted for the samples.

5.7.5 Chemical Oxygen Demand (Standard methods)

COD was estimated by closed reflux titrimetric method

Principle: Most types of organic matter are destroyed by a boiling mixture of chromic and sulfuric acids. A sample is refluxed with known amount of potassium dichromate and sulfuric acid. After digestion, the remaining unreduced dichromate is titrated with ferrous ammonium sulfate to determine the amount of K₂Cr₂O₇ consumed. The oxidizable organic
matter measured as oxygen equivalent, is proportional to the potassium dichromate consumed.

Reagents: Standard Potassium dichromate (0.0167M) digestion solution (having 167 ml conc H₂SO₄ & 33.3 g HgSO₄), {sulfuric acid reagent 5.5 g Ag₂SO₄ /Kg H₂SO₄}, standard ferrous ammonium sulfate titrant (FAS - 0.1M) and ferroin indicator solution.

Procedure: To 2.5 ml of appropriately diluted sample, 1.5 ml of digestion solution and 3.5 ml sulphuric acid reagent were added. The contents were refluxed for 2 hr in COD reactor (HACH COD reactor, Model 45600; manufactured by HACH Co. Iowa, USA). After cooling, residual dichromate was determined by titrating against 0.1M FAS using ferroin indicator, till the end point, a sharp color change from blue-green to reddish brown appeared. In the same manner, a blank containing the reagents and a volume of distilled water equal to that of the sample was refluxed and titrated. COD was calculated using the following relationship:

\[ \text{COD} = (\text{Blank reading} - \text{Sample reading}) \times \text{Molarity of FAS} \times 8000 \]

\[ \text{ML of sample taken} \]

5.8 Biomass

Principle: A rapid estimate of cell mass can be obtained by measuring the turbidity of a bacterial suspension. Turbidity measurements are generally made using a colorimeter or spectrophotometer, both of which
work by directing a light beam through the sample. The bacteria in the sample scatter the light beam and lower the intensity of the light beam coming through the suspension. At low bacterial intensities, there is a direct linear relationship between the number of bacteria and the amount of light scattered; thus as the number of bacteria increases, the turbidity of the suspension increases. At high bacterial densities, this relationship becomes nonlinear. Therefore, a standard curve must be constructed to determine the linear range for turbidity measurement for each organism being measured.

Procedure: Growth was followed by measuring the absorbance (OD$_{550}$) of the samples at room temperature in a spectrophotometer (Systronics Model 106, India). A standard growth curve was prepared by taking a known amount of culture (250 ml) grown on phenol (200 mg.L$^{-1}$), centrifuging (10,000 g/10 min) and harvesting the cells in the exponential growth phase. The cell pellet was then washed in distilled water and re-suspended in 50 ml of distilled water. Aliquots in the range of 0.5 to 2.5 ml were taken from the cell suspension, diluted to 5 ml and the absorbance at 550 nm was determined. For estimating the dry weight, 40 ml aliquot of the same cell suspension was filtered under pressure through a 0.45 µm millipore membrane that was previously dried (60 °C/1 h) and weighed. The membrane was dried at 60 °C for 24 hr, cooled to room temperature in a desiccator and weighed. A standard graph was
prepared by plotting absorbance against dry weight. The dry weight of unknown sample was obtained from the slope of the standard graph.

5.9 Scanning Electron Microscope

Granules for scanning electron microscopy (SEM) were prepared as per the procedures suggested by Glauert. The samples were prepared for SEM by fixing with glutaraldehyde, washing with phosphate buffer and dehydrating with acetone.

Reagents: (a) NaH₂PO₄ solution (0.2 M)
(b) Na₂HPO₄ (0.2 M)
(c) Phosphate buffer (0.2 M), pH = 7
(d) Phosphate buffer (0.1 M), pH ≈ 7
(e) Phosphate buffered 6 % glutaraldehyde fixative

Procedure: The granule was mixed with an appropriate amount of the fixative by gently tipping the tube and left for fixation for 1 hr at 20 °C, preferably in a refrigerator. The fixed sample was then washed in 0.1M phosphate buffer for 2 hr (preferably overnight). The buffer was changed thrice and the contents were kept at 4 °C during washing. The granule was then dehydrated by passing it through a graded series of solutions of increasing concentration (50, 70, 95 %) of the dehydrating agent (ethanol or acetone) in water for 10 min each and ending with absolute dehydrating agent for 15 min. During dehydration, each solution was removed carefully with a syringe and the next poured on. The samples
thus prepared were mounted on sample holders (Brass) and coated with silver in a sputter coating unit, and scanned using a scanning electron microscope (Model Jeol JSM840A, Japan) at an accelerating voltage of 5 kV-15 kV.

5.10 Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Suspended Volatile Suspended Solids

MLSS was calculated by taking 25 ml of the granular sludge in a silica crucible. These granules were dried at 105 °C for 12hr and the allowed to cool at room temperature. Difference of empty weight of the crucible and the dried weight along with the granules gives the MLSS concentration.

To calculate the MLVSS same crucible is kept in the Muffle furnace at 600 °C for 2hr. It is then allowed to cool and the difference in weight gives the MLVSS concentration.

References
