Materials & Methods......
4. Materials and Methods

4.1 Chemicals and Glassware

Glassware used in the present study were manufactured by M/S Borosil glassworks Ltd. (Mumbai, India) and marketed under the brand name 'Borosil'. They were washed with Liquid soap (Labolene, Qualigens, India) and finally rinsed with distilled water. All chemicals used were of analytical grade. Few of them were purchased from Sigma Chemicals, USA. Bacteriological media were purchased from Himedia Laboratories Ltd., Mumbai (India).

4.2 Sources of Inocula

4.2.1 Source of inoculum for anaerobic dye decolorization studies

Sludge from Upflow Anaerobic Sludge Blanket Reactor (UASB), receiving Kanpur city domestic as well as tannery wastewaters, was used as a source inoculum.

4.2.2 Source of inocula for the development of aromatic amine degrading enrichment cultures

Various sources of inocula were used for the development of aerobic enrichment cultures. They included

♦ Activated sludge from the full scale treatment plant located at Jajmau, Kanpur, receiving domestic wastewater.

♦ Sludge from the primary and secondary (biological) treatment units from a dye processing industry.

♦ Garden soil from IIT campus.

♦ Soil from tannery sludge disposal site.

♦ Soil from the vicinity of dye units from Sanganeeer, Rajasthan
4.3 Bacteriological Media

Medium used was dependent on the specific study. For anaerobic dye decolorization, stock nutrient medium (NM) was prepared and required volume was added to the influent. Many growth media were used for studies on the aerobic degradation of aromatic amines. Composition of these media are given in Sections 4.3.1 to 4.3.3.

4.3.1 Nutrient Medium (NM)

Composition of NM is given in Table 4.1 (Kroeker et. al., 1979). As the nutrient requirement of anaerobic bacteria is generally low as compared to aerobic bacteria, influent for the anaerobic reactor was supplemented with 10ml NM/Litre.

Table 4.1: Synthetic nutrient media composition (Kroeker et. al., 1979)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>4000</td>
</tr>
<tr>
<td>MgSO₄. 7H₂O</td>
<td>126</td>
</tr>
<tr>
<td>CoCl₂. 6H₂O</td>
<td>36</td>
</tr>
<tr>
<td>FeCl₃. 6H₂O</td>
<td>864</td>
</tr>
<tr>
<td>CaCl₂. 6H₂O</td>
<td>600</td>
</tr>
<tr>
<td>Urea</td>
<td>4000</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>400</td>
</tr>
</tbody>
</table>
4.3.2 Growth medium for 4-aminobenzenesulfonate (4-ABS) degradation

Two different mineral media, MM1 and MM2, were used for the growth of aerobic microorganisms degrading 4-ABS. The composition of these media are given in Table 4.2 and 4.3 respectively.


<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration g/L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄. 2H₂O</td>
<td>2.00</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.00</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.50</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.06</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.25</td>
</tr>
<tr>
<td>CaCl₂. 2H₂O</td>
<td>0.035</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.010</td>
</tr>
<tr>
<td>Trace elements solution*</td>
<td>1ml</td>
</tr>
</tbody>
</table>

*Composition given in Table 4.4.

**Table 4.3 Mineral medium MM2 (Feigel & Knackmuss, 1988)**

<table>
<thead>
<tr>
<th>Stock Solution A (200ml)</th>
<th>Concentration g/L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 - ABS neutralized</td>
<td>5.0</td>
</tr>
<tr>
<td>K₂PO₄</td>
<td>8.0</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Stock Solution B (790ml)**

| MgCl₂. 2H₂O               | 0.1                             |
| Trace elements solution*  | 2.0ml                           |

**Stock Solution C**

(10ml acidified)

FeCl₂ 4.0gm/L, (pH < 2.0)

The stock solutions A, B and C were autoclaved and mixed together after cooling.
Table 4.4 Composition of trace element solution (Kneimeyer et.al., 1999)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration g/L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>1.050</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.015</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.050</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.095</td>
</tr>
<tr>
<td>NiCl₂·6H₂O</td>
<td>0.012</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0145</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.072</td>
</tr>
<tr>
<td>Na₂MoO₄·7H₂O</td>
<td>0.018</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.6</td>
</tr>
<tr>
<td>(pH 6.5)</td>
<td></td>
</tr>
</tbody>
</table>

For ammonia release studies, NH₄Cl was omitted in the culture medium and the trace element solution without EDTA was used. For determination of Sulphate release during growth, MgSO₄ and K₂SO₄ were replaced with MgCl₂ and KCl respectively.

4.3.3 Growth media for 5-aminosalicylic acid (5-ASA) degradation

To prevent auto oxidation of 5-ASA in the growth medium, the lag phase of the microorganisms must be kept as short as possible. Therefore cultures were grown with nutrient broth (0.65g/L) and 1mM 5-ASA and then transferred to mineral medium3 (MM3). The composition of the MM3 was the same as MM1, except yeast extract concentration was 100mg/L.
4.4. METHODOLOGY

4.4.1a Development of culture for anaerobic dye decolorization

Aspirator bottle, with 2L liquid volume, was used as the reactor for developing methanogenic cultures. 10% of the anaerobic sludge, from a full scale UASB plant treating Kanpur city domestic wastewater and tannery effluent, was used as the seed inoculum. Feed solution consisted of 700mg/L glucose, 20ml of bicarbonate (50g/L) and 10ml of NM per liter of tap water. The reactor was operated in a once fed mode with an hydraulic retention time (HRT) of 3.3 days. After operating the reactor for 15-20 days in the 'once fed mode, the content were transferred to Sequential Fixed Film Anaerobic Batch Reactor (SFABR).

4.4.1b Sequential Fixed Film Anaerobic Batch Reactor for Dye decolorization

Experimental Setup:

The reactor consisted of cylindrical perspex column (dia 6.5cm, length 60cm). The reactor was filled with ceramic rings (inner dia. 3mm) upto 49cm. Contents of 2L reactor were transferred to the SBR. Total reactor volume was 1.7L and working volume was 1.37L. Feed solution (influent) entered vertically at the bottom through the sludge bed and the biogas excited through the port at the top (Fig. 4.1). Feed solution consisted of glucose (500mg/L), bicarbonate (20ml/Litre) from a stock solution of 50g NaHCO₃/L, NM (10ml per litre). pH of the feed was around 7.5 ± 0.3. Reactor was maintained at ambient temperature of 30 - 35°C. Gas volume was not measured in this study. Dye as well as its concentration was varied in the course of the study. At each concentration of specific dye, the reactor was operated for several
Materials and Methods

Fig. 4.1 Schematic diagram of the experimental setup of Sequential Fixed Film Anaerobic Batch Reactor (SFABR)
cycles (10 cycles). Before changing the dye in the feed solution, SFABR was operated for 10 days only with glucose in the feed. Dye concentration in the effluent was determined daily. pH, COD and VFA were periodically determined. Three model dyes were Acid orange 6 (AO 6, CI-547-57-9), Acid orange 7, (AO7, CI-15510) and Remazol Violet (RV, CI-27).

4.4.2 4-aminobenzenesulphonate (4-ABS) degradation

4.4.2.1 Development of enrichment culture

4-ABS degrading enrichment culture was developed from activated sludge derived from Kanpur city domestic wastewater treatment unit as the inoculum. 1ml of activated sludge suspension was added to 100ml of sterilized mineral medium (MM1) supplemented with 400mg/L (2.3mM) 4-ABS as growth substrate. When more than 80% 4-ABS was degraded, further enrichment was carried out by transferring 10% inoculum to 90ml portions of fresh medium, dispensed into 250ml erlenmeyer flasks, plugged with cotton and sterlised at 21lbs/15min. The following components were added prior to inoculation. 1ml of trace element solution per litre MM1 and required volume of 4-ABS solution from a 5g/L stock solution. Flasks were kept in a rotary shaker (120rpm) at 35°C.

Several inoculum sources such as activated sludge, sludge from biological treatment units from a dye processing industry or soil from the vicinity of dye units from Sanganeer, Rajasthan were tried to develop enrichments degrading 2-aminobenzenesulfonate (2-ABS) and 3-aminobenzenesulfonate (3-ABS).
**4.4.2.2 Isolation of pure cultures**

Isolation was carried out by serial dilution of the enrichment culture. Appropriate dilution were spread on 4-ABS supplemented agar plates (800mg/L) and incubated at 35°C. Bacterial colonies took more than 72hr for their growth on these plates. Hence 4-ABS (800mg/L) supplemented nutrient agar plates were used to get better growth in less time. Colonies were developed, on 4-ABS supplemented plates, within 24h. Morphologically distinct colonies were picked up and purified. A single colony was then inoculated into 10ml MM1 and 50mg/L 4-ABS. These flasks were kept in a shaking incubator at 100 - 120 rpm at 35°C. The flasks were monitored for growth and substrate disappearance. Immediately after substrate removal, this 10ml culture was transferred to 250ml conical flasks containing 90ml MM1 and 100mg/L 4-ABS. The culture purity was periodically checked by plating on both 4-ABS as well as nutrient agar plates.

**4.4.2.3 Characterization of pure cultures**

Isolated pure cultures were studied for their amine degrading ability under aerobic conditions. Promising strains PNS-1 and PNS-2 were then characterized based on standard morphological, physiological and biochemical tests (Cappucino, 1999).

**4.4.2.3a 16S r DNA sequence determination and analysis**

For 16S rDNA sequence analysis, cells were grown in 500ml MM1 supplemented with 800mg/L 4-ABS. Pelleted cells were suspended in 100% isopropanol and were then sent for 16SrDNA sequencing.

Genomic DNA was extracted and purified as described by Ausubel et al. (1992). PCR-mediated amplification of the 16S rDNA was carried out using the universal
primers 27f and 1525r according to Rainey et al. (1996). The same primers were also used for sequencing of the PCR products using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) with an ABI PRISM 377 DNA sequencer. Similarity searches were performed at NCBI using the BLAST program (Autschul, 1990), and a multiple alignment were constructed using ClustalX (Thompson, 1997). The sequence has been deposited in the Pubmed with accession number AY762361.

This work was carried out by Prof. N.K. Birkeland and his group in their laboratory at University of Bergen, Norway.

4.4.2.3b Gram Staining

Gram staining was done by using crystal violet, iodine, alcohol and safranin as per standard procedure.

4.4.2.3c Biochemical Tests

(a) Catalase Test

Principle: Catalase breakdowns H₂O₂ into O₂ and H₂O. Microorganisms having this enzyme produces bubbles of O₂, when it is introduced into H₂O₂ solution.

Procedure: A small volume of dense cell suspension was taken on a clean, dry glass slide with the help of sterile loop. Immediately a drop of 3% H₂O₂ was placed onto it. The evolution of bubbles (O₂) indicated a positive test for enzyme catalase.

(b) Spot Indole Test

Principle: Indole formed, from the action of tryptophanase on tryptophan, can be detected by its ability to combine with certain aldehydes to form a colored compound.
This can be visualized by production of blue-green end product, when the bacteria are rubbed on a filter paper impregnated with the substrate.

**Procedure:** Prepare Indole reagent by dissolving 1% p-dimethylamino-benzaldehyde in 10%(v/v) of conc. HCl. With the help of a sterile loop, a portion of colony was rubbed on a filter paper saturated with the reagent. Rapid development of blue-green color indicates a positive test for enzyme tryptophanase. Tryptophanase negative organisms remain colorless or turn slightly pinkish.

(c) Rapid Urease Test

**Principle:** Urease breakdown urea into ammonia. The alkalinity of ammonia changes the color of phenol red from yellow to red.

**Procedure:** Urea broth was prepared using 0.1g Yeast Extract, 0.091g Monopotassium Phosphate, 0.095g Disodium Phosphate, 20g Urea and 0.01g Phenol Red in 1000ml distilled water. Heavy suspensions of microorganisms were inoculated in 0.5ml aliquot. The sample was then incubated at 35°C and was observed at every 15, 30 and 60 minutes interval for a change of color. Urease positive organisms yield a bright pink or bright red color to broth whereas urease negative organism do not cause a change in the color of broth.

(d) Nitrate Reduction Test

**Principle:** Microorganisms, that possess nitrate reductase, can reduce nitrate to nitrite. Nitrite combines with naphthalene compound to form red colored end product. If the organism has further reduced nitrite to nitrogen gas, the test of nitrite will yield negative (colorless) result. An additional test for the presence of unreacted nitrate is
performed by using metallic zinc. With the addition of Zinc, a negative test will yield a red color, indicating the presence of unreacted nitrate.

**Procedure:** Nitrate broth was prepared using 0.65 g nutrient broth, 0.05 g KNO₃, and 0.05 g Agar in 50 ml of distilled water. The strains were then grown in 5ml of nitrate broth solution and were incubated at 35°C for 24 to 48 hrs. Durham tube was placed in the tube to trap bubbles of nitrogen gas. Then 3 drops of Reagent A (made by dissolving 4 g of sulfanilic acid in 500 ml of distilled water) and 3 drops of reagent B (made by dissolving 3 g of N-1-Naphthyl ethylenediamine dihydrochloride in 500 ml of 5M acetic acid) were added. Production of red color within 30 minutes indicates presence of enzyme nitrate reductase. The presence of unreduced nitrate can be detected by addition of a pinch of Zn powder to the broth. Nitrate reductase positive organisms either yield red color after addition of reagent A and B or remain colorless even after addition of zinc. Nitrate reductase negative organisms show no color after addition of reagents A and B but turn red with zinc.

**(e) Spot Oxidase Test (Kovac's Method)**

**Principle:** The enzyme cytochrome oxidase can oxidize substrate tetramethyl-p-phenylenediamine dihydrochloride to indophenol (colored end product). The purple colored end product will be visible if a small amount of growth from a strain that produces the enzyme is rubbed on substrate impregnated filter paper.

**Procedure:** A filter paper was moistened with a solution of 1% tetramethyl-p-phenylene diamine hydrochloride. A portion of colony was rubbed on this moistened filter paper. A change in color to blue or purple within 10 seconds indicates presence of enzyme cytochrome oxidase.
(f) Rapid Carbohydrate Fermentation Reaction Test

**Principle:** When organisms metabolize carbohydrate with subsequent changes in pH, the phenol red indicator changes from red to yellow. The presence of buffer controls the pH change and the heavy inoculum allows rapid detection constitutive enzymes.

**Procedure:** Buffered indicator was prepared using 0.005 g H$_2$PO$_4$, .02 g of K$_2$HPO$_4$, 0.4 g KCl and 0.002 g Phenol Red in 50 ml of distilled water. The pH was adjusted to 7 by using NaOH. Then the buffered indicator was passed through a 0.45µ diameter pore size membrane filter. Carbohydrate stock solution was prepared using 25 g/L glucose or sucrose. To 5ml of indicator buffer solution, 2ml of carbohydrate stock solution was added along with heavy suspension of pure culture of organisms. The tubes were then incubated at 35°C for 4 hrs. Positive organisms show a change in color from red to yellow within 30 minutes.

### 4.4.2.4 4-ABS degradation and Growth of Strain PNS - 1 and PNS-2

4-ABS degradation kinetics were monitored at different initial concentrations ranging from 100 - 5000mg/L (0.58-28.9mM). Aliquots were removed at different time intervals. Biomass build up was measured at 555nm after vortexing the sample for 1min. The sample was then centrifuged at 1150xg and filtered through 0.45µm filter membrane. 4-ABS consumption rates at different initial concentrations were calculated by plotting the concentration versus time and fitting the data to a linear regression model.

### 4.4.2.5 Determination of kinetic constants

The kinetic constants for 4-ABS degradation was determined by performing a number of runs in the concentration range of 100-2500mg/L (0.58-14.5mM). Biomass
increase was monitored periodically by measuring the optical density at 555nm so as to get as many as data points as possible.

4.4.2.6 Studies with dense cells suspension

Two different sets of experiments were tried.

(a) Cells grown in 4-ABS (800mg/L) MM1 were harvested in the exponential growth phase (O.D.<sub>555</sub> - 0.453) by centrifugation (10min, 10,000g, 4°C). The cell pellet was then washed in 0.7% NaCl solution and resuspended in half the original volume containing 100mg/L 4-ABS. Aliquots were periodically withdrawn and centrifuged in a table top centrifuge (10min, 1150xg) to separate the cells. UV spectra of the supernatant was recorded between 200-400nm.

(b) Effect of Inhibitors: Cells grown in 4-ABS supplemented (800mg/L, 300ml) MM1 were harvested in late exponential phase (O.D.<sub>555</sub>-0.785) by centrifugation (10min, 10,000xg, 4°C). The cell pellet was then washed in phosphate buffer (0.05M, pH - 7.0) and resuspended in 100ml of phosphate buffer. This was divided into five portions of 20ml each. 10ml phosphate buffer containing 4-ABS (1200mg/L) and different inhibitors such as 2,2 bipyridyl (3mM), Iodoacetamide (0.21mM), o-phenanthroline (3mM) and 8- hydroxyquinoline (3mM) were added to each flask. Different concentrations of 2,2 bipyridyl (0.05mM, 1mM, 2mM & 5mM) were also tried. A flask having final 4-ABS concentration of 400mg/l only was used as control.

4.4.2.7 Induction of 4-ABS degradation

Strain PNS-1 was grown in two 250ml flasks (100ml medium) in MM1 with succinate (1.9mM) upto an absorbance of 0.6 -0.65 under conditions described above. Cells were harvested by centrifugation at 10,000 rpm at 4°C and the cell pellet from
one flask was resuspended in 100ml sterile MM containing only 4-ABS (2.3mM) whereas the other received 4-ABS (2.3mM) and chloramphenicol (125mg/L). The flasks were kept in shaking conditions (120rpm) in an incubator. Degradation of 4-ABS with time was monitored. One control flask with 4-ABS pregrown cells instead of succinate was also included.

**4.4.2.8 Growth of Strain PNS-1 on other monocyclic aromatic compounds**

The ability of 4-ABS degrading strain PNS-1 to utilize other aromatic carbon sources, as growth substrate, was determined by inoculating 4-ABS grown cells into minimal medium (MM1) containing 2-aminobenzenesulphonate (2-ABS), 3-aminobenzenesulphonate (3-ABS), protocatechuate (3,4-dihydroxybenzoate, PCA), pyrocatechuate (2,3-dihydroxybenzoate, PYA), p-hydroxy benzoate (PHBA) and 5-sulphosalicylate (5SA) as sole carbon sources. Growth response was studied at two initial concentrations. Substrate concentration and biomass growth were monitored periodically. Batch studies were also conducted with mixed substrates where 4-ABS was one of the components. Culture conditions in all these studies were similar to those described earlier in section 4.4.2.4

**4.4.2.9 Protocatechuate 3, 4 dioxygenase assay**

Cultures were grown on 800mg/L aromatic carbon sources upto late exponential phase and cells were separated by centrifugation on Eppendorf centrifuge (5417R, 5810R) at 10,000g for 20min. The cell pellet was then suspended in 5ml of 50mM phosphate buffer (pH - 7.0). Crude extract was prepared by alternate freezing and thawing of cell suspension by plunging the vials into liquid nitrogen for 30sec and
transferring immediately to a water bath maintained at 37°C for 90sec. The thawing should be rapid in order to avoid the crystal formation. The freezing followed by thawing was carried out thrice. Extracts were stored on ice until used. Protocatechuate 3,4-dioxygenase was determined as per the procedure described by Stainer & Ingrahim (1954). The assay mixture consisted of 0.33mM protocatechuate, 33mM Tris - HCl buffer (pH - 8.0) and 50 - 200µl of crude extract in a total volume of 3.0ml. The enzyme activity was measured at 24°C by following the decrease in absorbance at 290µm which is due to substrate disappearance. One unit of enzyme is defined as the amount that oxidizes 1µmole of PCA per minute at 24°C. The molar extinction coefficient of substrate and product at 290nm are 3890 and 1590 respectively in a neutral solution. Protein content was measured using Folin's reagent.

4.4.2.10 Screening for the presence of Plasmid

Plasmid detection was carried out by employing modified alkaline lysis method (Sambrook and Russel, 2001 and Birnbiom, 2001). The methodology is based on the fact that the alkali breaks the cell wall and high salt solution precipitates the alkali and SDS. This also traps the chromosomal DNA. The plasmid DNA thus gets separated based on its small size. 4-ABS degrading strain PNS-1 grown on 800mg/L 4 - ABS (250ml) was used for plasmid detection. Cells were pelleted at 7000rpm at room temperature for 10min in eppendorf centrifuge 5810R (rotor no. 050720). The details of the experimental protocol is given in Table 4.5
4.4.2.11 Chloramphenicol treatment of 4-ABS degrading strain PNS-1 for amplification of low copy number plasmid

Addition of antibiotic chloramphenicol to bacterial culture medium can be used to amplify the copy number of plasmid. Chloramphenicol inhibits bacterial protein synthesis and thus bacterial chromosomal replication. Because the plasmid replicon does not require any newly synthesized proteins for replication, it continues to replicate in the presence of the drug (Qiang Zhou, 1992-2004). Following procedure was used. 30ml culture was inoculated into 500ml of L.B. broth (10mM NaCl, 0.5% (w/v) yeast extract and 2% (w/v) tryptone) and was grown for exactly 2.5 hr. at 37°C. 2.5ml of chloramphenicol solution was then added to a final concentration of approximately 170µg/ml. (Stock 34mg/ml in ethanol). The culture was incubated for an additional 10-16hr at 37°C. The bacteria were then ready for harvesting and plasmid purification. Method used for plasmid detection/isolation is given in Table 4.5

4.4.2.12 Immobilization of Strain PNS-1 by gel entrapment

The alginate entrapment of bacterial cells was performed as per the method described by Kierstan & Coughlan (1985). 4% alginate was prepared in distilled water by boiling and autoclaved for 15min. Bacterial cell pellet was suspended in modified MM1 (Table 4.6) so as to have O.D. 555 nm in the range of 12.5. Alginate solution and cell suspension were mixed in an equal ratio (v/v) and the mixture was extruded drop by drop into a cold and sterile 0.2M CaCl₂ solution (or BaCl₂) through a sterile 5ml pipette. Gel beads of approximately 3mm diameter were obtained. The beads were hardened by leaving the beads in CaCl₂ (or BaCl₂) solution for around 45min. with
Table 4.5 Alkaline Lysis Method (Maxi Prep)

<table>
<thead>
<tr>
<th>STEP</th>
<th>DETAILS</th>
</tr>
</thead>
</table>
| ♦ Pelleted cells resuspended in autoclaved alkaline lysis solution - I  
(50mM glucose, 25mM Tris, 10mM EDTA, pH - 8.0) | 750µl |
| ♦ Added freshly prepared alkaline lysis solution - II  
(2N NaOH, 1.0% SDS) | 1.25ml |
| ♦ Mixed by gentle inversion 5 - 7 times and incubated in ice for 10min. (The initially clear solution begins to get cloudy as SDS precipitates) | |
| ♦ Neutralized by adding alkaline lysis solution - III  
(3M Potassium acetate, pH - 4.8) | 1.25ml |
| ♦ Mixed by gentle inversion 5-7 times and incubated on ice for 10min. | |
| ♦ Centrifuged at 6000rpm for 10min. Removed all the cell debris. Transferred the supernatant to a fresh 15ml tarson tube. | |
| ♦ Added RNase (DNase free) solution. Incubated at 37°C for 20min. | 20µg/ml |
| ♦ Extracted twice with chloroform. | 1.5ml |
| ♦ Layers were mixed by hand for 30sec after each extraction. | |
| ♦ Centrifuged at 6000rpm for 2min. | ≈ 650µl in each tube |
| ♦ Transferred upper aqueous phase to a 1.5ml microfuge tube. | 850µl |
| ♦ Precipitated with 100% Ethanol. | |
| ♦ Decanted the supernatant. | |
| ♦ Dried it completely on paper towel by inverting the tube for 10 - 20min. | |
| ♦ Resuspended in 30µl autoclaved distilled water. | |
| ♦ Examined by Agarose gel | |
continuous stirring. Then the beads were transferred to modified MM1 with 200mg/L 4-ABS and stored at 4°C for further use.

(a) Batch Studies on 4-ABS degradation with immobilization cells

Batch degradation studies were conducted with free and immobilized cells. 2ml of cell suspension (O.D.\textsubscript{555 nm} -12.5) was added to 22.5ml of modified MM1 having an initial 4-ABS concentration of 200mg/L (1.15mM) and 400mg/L (2.3mM). The flasks were placed in an orbital shaker at 35°C at 120rpm. Aliquots were removed periodically for 4-ABS analysis. For immobilized cell, 1.25gm wet alginate beads (=48 beads derived from 2ml cell suspension) were suspended in 25ml modified MM1 having 4-ABS in the concentration range of 200 - 800mg/L. Experimental conditions were similar to those described earlier for free cells.

(b) Repeated Batch Degradation of 4-ABS

To assess the reuse potential of immobilized biocatalyst, repeated batch studies were carried out under identical experimental conditions described for the first cycle. After complete degradation of 4-ABS, the spent medium was decanted, beads were washed with sterile modified MM1 and transferred to fresh modified MM1 containing 400mg/L (or 800mg/L) 4-ABS.

c) 4-ABS degradation in Packed bed reactor

The feasibility of using a packed bed column for the biodegradation was studied under semicontinuous conditions. A cylindrical perspex column (35.5 x 3.3cm) was used as the reactor. Total reactor volume was 200ml. Glass beads were placed at the bottom. Porous glass frit was placed on the glass beads. Alginate beads were filled
upto 30cm height. Working volume and void volume of the reactor were 170ml and 62ml respectively. The sterile modified MM1, with varying 4-ABS concentration was passed through the column in an upflow mode at 72ml/h using a peristaltic pump for 12h each day. Feed inlet was provided at the bottom of the column and aeration was provided from an aquarium pump through the sidearm located at bottom of the column. Effluent was taken out from the outlet provided just above the packed bed.

Table 4.6: Composition of Low phosphate medium (Modified MM1)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>g/L distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>0.125</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.125</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.500</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>0.060</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.250</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.050</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.010</td>
</tr>
</tbody>
</table>

pH - 7 ± 0.2

4.4.3 5-aminosalicylate (5-ASA) degradation:

4.4.3.1 Development of enrichment culture

Aerobic enrichment culture degrading 5-ASA was developed by inoculating 5-ASA (1mM) supplemented 0.65g/L nutrient broth with the inoculum derived soil/sludge from the vicinity of tanneries (1gm/100ml) or activated sludge slurry (10ml/100ml). Flasks were incubated at 35°C on a rotary shaker at 120rev/min. Most of the studies
were carried out using above stated conditions. In few experiments, nutrient broth pregrown cultures were transferred to MM3 (Table 4.5)

4.4.3.2 Isolation of pure cultures

Isolation was carried out by serial dilution of the enrichment culture. Appropriate dilution were spread on 5-ASA (1mM) supplemented nutrient agar plates and incubated at 35°C in the dark. Colonies developed on 5-ASA supplemented plates within 24 hours.

4.4.3.3 Characterization of pure cultures

Characterization of pure cultures was carried out as per procedures described in 4.4.2.3.

4.4.3.4 5-ASA degradation and Growth

5-ASA degradation kinetics was monitored at three different initial concentrations (100-300mg/L) using MM3. Aliquots were withdrawn at different time intervals, centrifuged at 1150xg and spectrophotometrically monitored for decrease in absorbance at 330nm. Few experiments were also performed using same 5-ASA concentration (1mM) but different media composition. Growth was monitored at 555nm after vortexing the sample for 1min.

4.4.3.5 Studies with dense cells suspension

Cells were grown in 250ml conical flasks on (0.65g/L) nutrient broth. On complete substrate consumption, flasks were respiked with 1mM 5-ASA. These cells were again checked for substrate depletion and harvested by centrifugation (10min,
10,000xg, 4°C). The cell pellet was then washed in MM1 without NH₄Cl and resuspended in same medium (O.D₅₅₅-1.308) having 1mM 5-ASA. Aliquots were periodically withdrawn, centrifuged in a tabletop centrifuge (10min, 1150xg) and filtered through 0.45µfilter paper to remove the cells. UV spectra of the filtrate was recorded between 200-400nm. Ammonia release was also monitored by Nesslerization.

4.4.4 Aniline (AN) degradation
4.4.4.1 Development of enrichment culture
Activated sludge was used as the source inoculum for the development of enrichment with aniline as the sole organic carbon source. Culture conditions were similar to those described in 4.4.2.1. MM1 was used as the growth medium.

4.4.4.2 Aniline degradation and Growth
Aniline degradation kinetics was monitored at different initial concentrations ranging from 200-1000mg/L. Aliquots were removed at different time intervals. Biomass build up was measured at 555nm after vortexing the sample for 1min. The sample was then centrifuged at 1150xg and filtered through 0.45μm filter membrane. Aniline consumption rates at different initial concentrations were calculated by plotting the concentration versus time and fitting the data to a linear regression model.

4.4.5 6-aminonaphthalene-2-sulfonate (6AN2S) degradation
4.4.5.1 Development of enrichment culture
Enrichment for 6AN2S degraders was attempted in flasks as well as in open aerobic reactor. Enrichment procedure, for flasks with sterilized medium, was similar to that
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described for 4-ABS, except that 200mg/L 6AN2S was used as carbon source. Mineral medium used had high concentrations of phosphate. An aerobic batch reactor (2L) was used for this study. Total liquid volume in the aerobic reactor was 1L. Mixed inocula consisting of activated sludge from the full scale unit treating Kanpur city domestic wastewater and sludge from biological treatment units of a dye processing industry were used for the development of aerobic enrichment culture. 1L MM (Section 5.2.3.2) having 200mg/L 6A2NS was initially used for this enrichment process. Aliquots were removed every 24h and checked for substrate removal. 500ml content was daily removed and replaced with an equal volume of the fresh medium having same composition as mentioned above. Aeration was provided by an aerator and diffuser.

4.5 Analytical methods

Various analytical techniques used in this investigation are presented in the following subsections. In general, standard techniques as detailed in Standard Methods (APHA, 1992) have been followed unless otherwise mentioned. Liquid samples were filtered through 0.45µ filter for the analysis of soluble components.

4.5.1 pH

pH was measured using a digital pH meter (Systronics model MKVI, India)

4.5.2 U.V. Spectrophotometry

UV spectrophotometry was used for determining dye decolorization as well as for the estimation of 4-ABS, 2-ABS, 3-ABS, 5-ASA, aniline, 6AN2S and other compounds used in this study. U.V. spectra of the appropriately diluted samples of culture
supernatants were recorded. $\lambda_{\text{max}}$ for these analytes are given in Table 4.7. Calibration graphs were prepared in appropriate concentration range.

Table 4.7: Compounds along with their $\lambda_{\text{max}}$.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO6</td>
<td>429nm &amp; 248nm</td>
</tr>
<tr>
<td>AO7</td>
<td>483nm, 227nm &amp; 305nm</td>
</tr>
<tr>
<td>Remazol violet</td>
<td>560nm and 320nm</td>
</tr>
<tr>
<td>2-ABS</td>
<td>238nm</td>
</tr>
<tr>
<td>3-ABS</td>
<td>238nm</td>
</tr>
<tr>
<td>4-ABS</td>
<td>248nm</td>
</tr>
<tr>
<td>5-ASA</td>
<td>330nm in Nutrient broth</td>
</tr>
<tr>
<td>6AN2S</td>
<td>242nm</td>
</tr>
<tr>
<td>Aniline</td>
<td>230nm</td>
</tr>
<tr>
<td>PHBA</td>
<td>246nm</td>
</tr>
<tr>
<td>PCA</td>
<td>249nm and 287nm</td>
</tr>
<tr>
<td>PYA</td>
<td>239nm and 305nm</td>
</tr>
<tr>
<td>5-sulfosalicylic acid</td>
<td>239nm and 299nm</td>
</tr>
</tbody>
</table>

4.5.3 High Performance Liquid Chromatography (HPLC)

Principle: In HPLC, eluent from the solvent reservoir is pressurized and pumped through the chromatographic column. A mixture of solutes injected at the top of the column is separated into the components on traveling down the column and the individual solutes are monitored by the detector and recorded as peak on the chart.
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recorder. The main components of HPLC are a high-pressure pump, a column/injector system and a detector.

**Reverse Phase Column:** Reverse phase columns address the widest range of applications of any HPLC column type. They are prepared by binding alkylsilane reagent (Octadecyl) to the base 5 µ spherical silica. When an unknown mixture is to be analyzed, a reverse phase column is often tried first. Retention in the reversed phase liquid chromatography depends on the relative hydrophobicity of the compound. As expected on a non-polar surface, the less polar the solute, the longer is the retention time. Polar functional group in a molecule tend to decrease the attraction on the reversed phase packing.

In this study, two solvent systems (a) methanol: H₂O: acetic acid [v/v/v] in the ratio 60:38:2 and (b) mixture of methanol (40%) and 0.05M phosphate buffer (60%, pH-6.5) were used for the separation of 4-ABS and other aromatic compounds on C₁₈ column. The flow rate of the solvent was always maintained at 0.5ml/min. Under the above conditions, with solvent (a) retention times of 4-ABS, PCA, PYA and PHBA were 4.7, 5.7, 7.6 and 5.8 respectively.

### 4.5.4 Electrophoresis

**Principle:** Electrophoresis is an analytical tool which is based on the migration of charged molecules to the electrodes of opposite polarity in an applied electric field. Many biological molecules carry an electric charge, the magnitude of which depends upon the molecule as well as on the viscosity, ionic strength and pH of the suspending medium i.e. buffer. Apart from these, other important factors affecting the migration of molecules are the interaction of migrating molecules with the support medium and shielding of molecules by buffer ions. Differential migration of bio molecules, by
selecting an appropriate medium and solid support in electrophoresis is extensively used for the analysis of their purity and size. Each molecule in a mixture is expected to have unique charge and size and its mobility in an electric field will therefore be unique. This forms the basis for the analysis and separation by all electrophoretic methods. The technique is especially useful for the analysis of amino acids, peptides, proteins, nucleotides, nucleic acids and other charged molecules.

(a) SDS - PAGE Gel Electrophoresis

Reagents: i) Solution (A) Acrylamide solution (30%): 29.2gm acrylamide and 0.8gm bisacrylamide was taken and dissolved in 100ml water.

ii) Solution (B) Lower Buffer (1.5M Tris buffer, pH 8.8): Dissolve 18.17gm of Tris and 0.1gm of SDS in distilled water. Adjust for pH 8.8 with HCl and made upto 100ml.

iii) Solution (C) Upper Gel Buffer (0.5M Tris buffer, pH 6.8): Dissolve 6.06gm of Tris and 0.4gm of SDS in water. Adjust for pH 6.8 with HCl and make upto 100ml.

iv) Solution (D) Ammonium persulphate (10%): Add 1ml of water to 0.1gm of ammonium per sulphate prepare just prior to use.

v) Preparation of SDS sample buffer: 20% (w/v) glycerol, 10% (w/v) 2 - mercapto ethanol, 4.6% (w/v) SDS, 0.12M Tris HCl (pH - 6.8) and 0.1% bromophenol blue.

vii) Electrophoretic buffer: Mix 10ml of 10% SDS solution to 3gm of Tris and 14.9gm of glycine and make upto 1000ml with water.
viii) **Staining and destaining Solution:** Add water to 2.5gm of coomassie brilliant blue, 500ml of ethanol and 100ml of acetic acid to make it upto 1000ml (Staining solution). Add water to 250ml of Methanol and 70ml of acetic acid. Make it upto 1000ml (destaining solution.)

**Procedure:** Gel solution for a 1mm thick gel

<table>
<thead>
<tr>
<th>Composition:</th>
<th>(Quantity; in ml)</th>
<th>(%; gel concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Separating Gel (12.5%)</strong></td>
<td><strong>Concentrating Gel (4.5%)</strong></td>
<td></td>
</tr>
<tr>
<td>Solution A</td>
<td>7.50ml</td>
<td>0.90ml</td>
</tr>
<tr>
<td>Solution B</td>
<td>4.50ml</td>
<td>-</td>
</tr>
<tr>
<td>Solution C</td>
<td>-</td>
<td>1.50ml</td>
</tr>
<tr>
<td>Solution D</td>
<td>0.07ml</td>
<td>0.018</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01ml</td>
<td>0.01ml</td>
</tr>
<tr>
<td>Water</td>
<td>6.00ml</td>
<td>3.60ml</td>
</tr>
</tbody>
</table>

Mix Solution A, Solution B (or Solution C) and water, add TEMED and solution D and gently mix. Immediately cast a gel.

**Sample preparation**

- Determine protein concentration in sample: 1-2mg/ml
- Mix with loading gel dye.
- Heat for 5 min. in a water bath to denature the proteins.
- Load 30µl of the prepared sample in the well.

Apply a voltage of 8 V/cm to gel. After the dye front has moved into resolving gel, increase the voltage to 15V/cm and run the gel until the bromophenol blue reaches the bottom of resolving gel.
(b) Agarose gel Electrophoresis

Agarose gel was made by dissolving 0.35g agarose powder in powder in 50ml TBE (Tris Borate) buffer. On cooling to nearly 50°C, 2.5 µl Ethidium bromide (10mg/ml) was added and swirl mixed gently. This was poured into a gel mould with its side taped. Well forming comb was placed 1mm above the plate surface near one edge of the gel. This comb was removed when the gel had solidified to leave "wells" for the DNA to be loaded. Loading of the DNA was done according to its concentration keeping the ratio of 0.5µg/mm lane width in mind. (Sambrook and Russel, 2001) A standard marker was also included in one lane as a size marker.

4.5.5 Chemical Oxygen Demand

Principle: COD was estimated by closed reflux titrimetric method. Most type of organic matter are destroyed by boiling mixture of chromic acid and sulphuric acid. A sample is refluxed with known amount of Potassium dichromate and sulphuric acid. After digestion, the remaining unreduced dichromate is titrated with Ferrous ammonium sulphate to determine the amount of Potassium dichromate consumed. The oxidizable organic matter measured as oxygen equivalent, is proportional to the potassium dichromate consumed.

Reagents: Standard potassium dichromate (0.0167 M) digestion solution (having 167 ml conc. H₂SO₄ and 33.3 g HgSO₄). Sulphuric acid reagent 5.5 g Ag₂SO₄/Per Kg H₂SO₄. Standard Ferrous Ammonium Sulphate Titrant (FAS-0.05M). Ferroin indicator solution.

Procedure: To 2.5 ml of appropriately diluted sample (50-150mg/L), 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
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by HACH co. Iowa, U.S.A). After cooling, residual dichromate was determined by titrating against 0.05 M 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:

\[
\text{COD (mg/L)} = \frac{(\text{Blank reading} - \text{Sample reading}) \times \text{molarity of FAS} \times 8000}{\text{ml sample taken}}
\]

4.5.6 Total Organic Carbon (TOC)

Description: Total Organic Carbon (TOC) analysis is a well defined and commonly used methodology that measures the carbon content of dissolved and particulate organic matter present in the water. TOC analysis consists of inorganic carbon removal, oxidation of the organic carbon into CO₂, and quantification of the CO₂. TOC measurement yields a valuable information on the mineralization of organic compounds by microorganisms. In this study, TOC analysis (model TOC-VCPN, Shimadzu) was carried out to ascertain the mineralization of aromatic amines.

4.5.7 Determination of Total Amines using \textit{p-}
dimethylaminobenzaldehyde

Method: To 0.2 ml of culture supernatant, 0.8 ml of distilled water and 0.05 ml of 1M HCl were added, and then 3ml of ethanol, 0.5ml of 5\% \textit{p-}
dimethylaminobenzaldehyde in ethanol were added. After 10min, the absorbance was measured at 440nm. in a digital spectrophotometer (Oren et. al, 1991). Calibration graph was prepared with required amines in the concentration range of 0 - 100mg/L.
4.5.8 Ammonia

**Principle:** Ammonia was measured colorimetrically by Nesslerization. Nessler’s method is sensitive to 20µg NH$_3$-N/L under optimum conditions and may be used up to 5mg NH$_3$-N/L. turbidity, color, and substances precipitated by hydroxyl ion such as magnesium and calcium, interfere and are 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 intensity of yellow color formed in the concentration range of 0-5mg/L NH$_3$, measured at 440nm followed Beer's Law.

**Reagents:**
(a) Nessler's reagent (commercially available)
(b) Rochelle salt solution (50g sodium potassium tartarate in 100 ml water is boiled and reduced to 70mL followed by making it up to 100mL)
(c) Stock ammonium solution: anhydrous NH$_4$Cl was dried (100°C) and 3.819g was dissolved in water and diluted to 1000mL; 1ml=1mg NH$_3$-N =1.22mgNH$_3$.
(d) Standard ammonium solution: stock ammonium solution, 10mL was diluted to 1000mL with water; 1mL=10µg NH$_3$-N=12.2µg NH$_3$

**Procedure:** Sample or a portion was diluted to 10mL with distilled water and to this, 1-2 drops of Rochelle salt solution added, 0.2mL Nessler's reagent was then added and samples were mixed thoroughly. After 10min, the absorbance was measured at 440nm against reagent blank. Calibration curve was prepared using NH$_4$Cl as standard in the concentration range, 0-5mg/L under conditions identical with those adopted for the samples.
4.5.9 Sulphate

Procedure described by Bertolacini and Barney (1957) was used for the determination of sulphate ion concentration in the culture fluid.

**Reagents:** Sodium sulfate (1g/L), 1mM Citric Acid, Ethanol and Barium chloroanilate

**Procedure:** Ethanol (12.5ml) was added to 7.5ml of culture supernatant and the solution was acidified with citric acid (1M) to pH 4. Following this, 150mg of Barium chloroanilate was added and the sample was then diluted with distilled water to a final volume of 25ml and shaken for 10min. Undissolved particles were removed by centrifugation. Photometric measurements were carried out at 530nm. The calibration curve was established in a similar way by using standard solutions of sodium sulfate in the concentration range of 0 - 666mg/L.

4.5.10 DNS Test

**Principle:** Several reagents have been employed which assay sugars by using their reducing properties. One of such compound is 3,5 dinitro salicylic acid (DNS) which in alkaline solution is reduced to 3-amino-5-nitro salicylic acid.

**Reagent:** The reagent was prepared by dissolving 1 g of 3,5-dinitro salicylate, 30 g of sodium potassium tartarate and 1.6 g of sodium hydroxide in water and making up to 100 ml. A standard solution of glucose was prepared at a concentration of 1g/L.

**Procedure:** Different volumes of glucose solution ranging 0.25 to 2.0 ml are pipetted out into various tubes and the volume made upto 2 ml with addition of water. To each tube, 2.0 ml of the reagent was added. The tubes covered with foil paper and kept in boiling water for 10 minutes, after which they are cooled and diluted up to 10 ml of water. The orange red color found was measured at 540nm.
4.5.11 Determination of the total protein content of whole cells

**Principle:** Protein reacts with Folin's ciocaltaeu reagent to give a colored complex. The color so formed is due to the reaction of alkaline copper with protein as in biuret test and reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

**Reagents:**
1. NaOH solution: 2N NaOH [dissolve 8 gm NaOH in 100ml distilled water],
2. Reagent A: 13%w/v Na₂CO₃ [94ml], 4%w/v Sodium potassium tartrate [3ml], 2%w/v CuSO₄·5H₂O [3ml],
3. Folin's reagent: Commercially available, 1:1 dilution

**Procedure:** 0.25ml cell suspension was diluted with distilled water and made upto 1ml. 0.5ml NaOH (2N) was added to the cell mixture. The mixture was boiled at 100°C for 10min. so that maximum protein get denatured. After cooling 1.5ml reagent A was added. After 10min, 0.5ml Folin's reagent was added. Optical density at 625nm was measured after 30min. in UV-160A, Shimadzu spectrophotometer. A standard curve was prepared by plotting the absorbance versus protein concentration.

4.5.12 Volatile Fatty Acids (VFA)

**Principle:** Direct titration method described by DeLallo and Albertson (1961) was used to determine the VFA. This involves titration of the samples with strong acid to pH 4.3 which gives alkalinity due to bicarbonate, VFA and phosphate. When the sample pH is reduced to 3.3 bicarbonate ions will be converted to carbonic acid and subsequent boiling of the sample removes all of the carbonic acid as carbon dioxide. The back titration from pH 4.0 to 7.0 measures the alkalinity due to organic acids. The conversion factor for the determination of VFA from volatile acids alkalinity depends on the proportion of acid which is titrated between pH 4.0 to 7.0.
Procedure: A 25ml of centrifuged sample was titrated to a pH of 4.3 with 0.1N sulphuric acid. The volume of acid used was noted and titrated was continued to pH of 3.5-3.3. The sample was gently boiled for three minutes and cooled in a water bath to room temperature. It was then titrated against 0.05N NaOH up to pH 4.0. After noting the burette reading, the titration was continued to pH 7.0 and final reading was noted. VFA concentration was calculated as follows:

The multiplying factor 1.5 takes care of the conversion of CaCO₃ alkalinity to VFA expressed in terms of CH₃COOH and the assumption that 80% of VFA is titrated from pH 4.0 to 7.0.

\[
\text{VFA alkalinity, mg/L as CaCO}_3 = \frac{(\text{ml 0.05N NaOH x 2500})}{\text{ml sample}}
\]

\[
\text{VFA, mg/L as CH}_3\text{COOH} = \text{VFA alkalinity x 1.5}
\]

The multiplying factor 1.5 takes care of the conversion of CaCO₃ alkalinity to VFA expressed in terms of CH₃COOH and the assumption that 80% of VFA is titrated from pH 4 to 7.

4.5.13 Biomass

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

Procedure: Growth was followed by measuring the absorbance (O.D_{555}) of the
sample at room temperature in a spectrophotometer (Systronics model 106, India).
Standard growth curve was prepared by taking a known amount of culture (200ml)
grown on 4-ABS (800mg/L), centrifuging (10,000g, 10min.) and harvesting the cells
in the exponential growth phase. The cell pellet was then washed in 0.9% NaCl and
resuspended in 50ml of minimal media. Aliquots in the range of 2ml - 8ml were taken
from the cell suspension. Diluted to 10ml and the absorbance at 555nm was
determined. For estimating the dry weight 25ml aliquots of the same cell suspension
was filtered under pressure through a 0.45µm millipore membrane that was
previously dried (60°C for 1h) and weighed. The membrane was dried at 60°C for
24h, cooled at room temperature in a dessicator 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. An O.D_{555} of 1 was equal
to 340 cell dry weight per liter.