CHAPTER - II

METHODS AND MATERIALS
Organism and Maintenance

The bacterial strains *Pseudomonas* (diff) spp. and *Pseudomonas* spp. B-2 were isolated by standard enrichment techniques (1), using benzoate and 2-chlorobenzoate (2-CB) as sole source of carbon and energy. *Pseudomonas* (diff) spp. was isolated from a complex petrochemical sludge provided by M/s Indian Petrochemical Corporation Ltd. (Baroda) whereas *Pseudomonas* spp. B-2 was isolated from the soil of a pesticide manufacturing factory (Baroda). Both the bacteria produced a fluorescent blue pigment and were identified as belonging to the genus *Pseudomonas*. The organisms were maintained on agar slants of Chakraborty’s medium (described below) containing 0.2% (w/v) benzoate or 0.1% (w/v) 3-chlorobenzoate for *Pseudomonas* (diff) spp. and 0.2% (w/v) 2-chlorobenzoate for *Pseudomonas* spp. B-2.

Growth Studies: An inoculum of cells was prepared by growing the organisms in Chakraborty’s medium (2) containing in gm/litre: \((\text{NH}_4)_2\text{SO}_4 2.0; \text{K}_2\text{HPO}_4 5.8; \text{KH}_2\text{PO}_4 4.5; \text{MgCl}_2 0.16; \text{MnCl}_2 1 \text{ mg}; \text{NaMoO}_4 2.0 \text{ mg}; \text{CaCl}_2 20 \text{ mg} \) and distilled water to a final volume of 1.0 litre and pH of the medium was found to be 7.0. 0.1% (w/v) benzoate, or 2-fluorobenzoate were added as a sole source of carbon and the pH was adjusted to 7.0, for *Pseudomonas* (diff) spp. 3-chlorobenzoate (3-CB) was added at a concentration of 0.1% (w/v) along with 0.05% (w/v) yeast extract. All the other carbon sources were added minus the yeast extract. For the strain *Pseudomonas* spp. B-2 all hydrocarbons were added at a concentration of 0.2% (w/v) without yeast extract. The media were sterilized at 10 lb pressure for 20 mins. 10 ml of exponentially growing culture was added to 100 ml hydrocarbon medium in a 250 ml flask and the flasks were incubated at 28°C ± 2°C on a rotary shaker (150 rpm). Samples were removed at various time intervals and growth was measured turbidometrically by taking the optical density at 540 nm on a Spectronic-20 (Bausch and Lomb).
Luria Bertani (LB) Medium: Bactotryptone 10.0 gm; yeast extract 5.0 gm; NaCl 5.0 gm; dissolved in distilled water to make final volume to 1 litre, pH 7.2.

The concentration of free chloride / fluoride ions in the medium were determined by using ion selective chloride / fluoride electrodes, (ORION Research Inc. Cambridge, Massachusetts), which had been calibrated with different concentration of sodium chloride / sodium fluoride (10^{-4} to 10^{-2} M) in distilled water (D/W).

**Identification and Characterization of Metabolites:**

The supernatant culture fluid separated by centrifugation was acidified to pH 2.0, with sulphuric acid and extracted twice with diethyl-ether and the organic phase was dried over anhydrous sodium sulphite.

**Thin Layer Chromatographic (TLC) Analysis:** The organic layer was evaporated to a small volume and spotted on 0.25 mm thick silica gel G (Ranbaxy) plates for qualitative analysis. The mobile phase used was benzene : toluene : acetic acid (2:2:1) and the compounds were located by iodine vapours as well as by spraying colour reagents. Preparative TLC for purification of metabolites was carried out on 0.5 mm thick silica gel G plates. The mobile phase was the same as described above. The bands obtained by preparative TLC were eluted with diethyl ether and the organic phase was evaporated to dryness. This was used for further analysis.

The metabolites of 2-fluorobenzoate degradation were also purified by column chromatography using silica gel G (less than 0.08 mm finer than 200 mesh, Acme India). The metabolites were eluted by the solvent system of chloroform : acetone (8:2). The metabolites were dried and the corresponding purified metabolites were used for further studies.
Gas Liquid Chromatographic (GLC) Analysis: The crude extract or the eluted metabolites were trimethyl silylated with N,O-bis-(trimethyl silyl)-acetamide and analysed on a GLC chromatograph (CIC, Baroda) equipped with a flame ionization detector (FID), with an SE-52 column operated at 190°C. The compounds were identified by comparing their retention times with the authentic standard.

High Pressure Liquid Chromatography (HPLC) Analysis: Concentrations of substrate and metabolites in the culture fluid or ether extracts were determined by HPLC with a 30 cm x 3.9 cm Zorbax ODS (C\textsubscript{18} octadecyl trichlorosilane chemically bonded to borosil) reverse phase column and the operation was carried out at 28°C ± 2°C. Peaks were assigned to the metabolites after co-chromatography with authentic compounds and also by recording the UV spectra when the flow was stopped during maximum absorbance with use of a variable wavelength spectrophotometer detector model, Shimadzu LC-4A liquid chromatography (Japan).

Detection of chlorocatechol was done in a solvent system of methanol : water : acetic acid (60:40:5) with a flow rate of 0.5 ml / min and the spectrophotometric detector at 287 nm. Intermediates of 2-fluorobenzoate metabolism were separated in a system of water : methanol (6:4) and a flow rate of 0.8 ml/min. 2-chlorobenzoate pathway metabolites were separated in a solvent system of water : methanol (6:4) adjusted to pH 5.0 with orthophosphoric acid, the flow rate was maintained at 0.8 ml/min. For the identification of catechol as an intermediate in 2-CB metabolism, HPLC analysis was carried out in a mobile phase of water : methanol (1:1) with the flow rate of 1 ml/min.

Spectrophotometric Analysis: Purified metabolites were dissolved in methanol and were scanned on Shimadzu UV visible recording spectrophotometer UV-260, in the spectral range of 200-400 nm.
Methylation of Extract: The 3-CB metabolites were purified by TLC or column chromatography and the purified extract was methylated as described by Reineke et al. (3). Briefly, one gram of nitrosomethyl urea was dissolved in minimum amount of cold dry ether under cold conditions. To this was added 20 ml of 50% (w/v) KOH and shaken till yellow colour persisted. The yellow coloured etheral layer was then poured into the extract and the reaction was monitored by TLC till completion. After the reaction was complete, traces of acetic acid were then added to discharge the yellow colour. The mixture was washed with NaCl solution to remove the unreacted diazomethane and the etheral layer was concentrated. The derivatized metabolite was purified by preparative TLC and was then subjected to mass spectral analysis on Varian, Mat CH7 spectrometer (70 eV).

Gas Chromatography-Mass Spectra Analysis: The 2-fluorobenzoate metabolites were analysed on a V.G. Micromass 7070H with a 3% OV-17 column coated on chromosorb AW 60/80 mesh having 0.25 in. diameter and 6 ft. length glass column, operated at 170°C. A mass spectrum of 30 eV with a trap current of 200 microamps and an ion source temperature of 200°C was carried out of the gas chromatograph.

Preparation and Detection of 3,5-Cyclohexadiene-1,2-diol-1-carboxylic Acid (DHB):

Dihydrodihydroxybenzoic acid (DHB) were prepared biologically by using Alcaligenes eutrophus B9, a mutant strain as described by Reineke et al. (3). The strain Alcaligenes eutrophus B9 was a kind gift from Dr. G.D. Hegeman (Dept. of Microbiology, Indiana University, Bloomington, Indiana), and has been described by Johnson and Stanier (4). This strain is blocked in the conversion of DHB to catechol, since it is defective in the gene for DHB dehydrogenase and so accumulates DHB from benzoic acid. Alcaligenes eutrophus B9 and Pseudomonas (diff) spp. were grown on succinate medium (0.2% w/v)
and the cells were induced by the addition of 2 mM benzoate or 2 mM 2-fluorobenzoate. After incubation for various time intervals, the culture filtrate was extracted and the products analysed on HPLC at 266 nm (λ max of DHB = 266 nm).

**Respirometric Studies**:  

The rates of oxygen uptake were measured polarographically with a Gilson oxygraph model 5/6 (Gilson Medical Electronics, Inc. USA). Freshly harvested actively growing cells of *Pseudomonas* spp. B-2 grown on various hydrocarbons were washed twice in 20 mM phosphate buffer pH 7.0. Sufficient cells were then added to the reaction system (total chamber volume 2.1 ml) so as to obtain an optical density of 0.3 at 540 nm. The buffer and distilled water used were saturated with air. Endogenous oxygen uptake of cells was monitored for 10 min. after which the reaction was initiated by injecting 0.5 μmoles of assay substrate. Oxygen uptake rates were corrected for endogenous uptake. All operations were carried out at 28 ± 2°C.

**Preparation of Cell Free Extract (CFE)**:

a) **CFE of Pseudomonas (diff) spp.** : Exponentially grown cells on various carbon sources were harvested by centrifugation, washed twice with 20 mM phosphate buffer pH 7.2, and were subjected to repeated grinding in a chilled mortar and pestle with glass powder as abrasive, 20 mM Tris-HCl pH 8.0 containing 2 mM mercaptoethanol, was used as the extraction buffer, and this was centrifuged at 39,000 x g for 30 mins, at 4°C to remove the cell debris. The clear supernatant was designated as cell free extract (CFE).

b) **CFE of Pseudomonas spp. B-2** : Mid log phase cells were harvested by centrifugation and washed with 20 mM phosphate buffer pH 7.0. The cell paste was suspended in 20 mM Tris-HCl pH 7.0 and sonicated for 15 mins. in 30 sec.
burst with 30 sec. rest using an ultrasonic processor (Vibromec, India). The cell homogenate thus obtained was centrifuged at 39,000 x g at 4°C for 30 min. The clear supernatant was designated as the CFE.

For anaerobic incubation the reaction system was flushed with nitrogen and then incubated in anaerobic jars (Oxoid, England) to prevent entry of air during the period of incubation.

For whole cell dehalogenation activity, similar conditions were used except that the enzyme proteins were substituted by 10⁸ cells/ml.

Cell Free Extract Transformations: The cell free extracts were prepared as described above. For Pseudomonas (diff) spp., 20 ml reaction systems were set up using various cofactors. The concentrations of substrates and each cofactor used were follows: 2-fluorobenzoate, 0.005 M; FAD, 3 μmoles; NADH, 15 μmoles; NADPH, 1.5 μmoles; Boiled CFE was taken as control. To all the reaction systems 1.2 mg of the antibiotic tetracycline was added to avoid contamination.

The CFE of Pseudomonas spp. B-2 was prepared using 20 mM phosphate buffer (pH 7.0) or 20 mM Tris-HCl (pH 7.0) along with and without 2 mM dTT or 2-mercaptoethanol. 25 ml reaction systems were set up, and the concentrations of substrate and each of the cofactors used were as follows: 2-chlorobenzoate, 0.5 mM; FAD, 3 μmoles; NADH, 15 μmoles, Boiled CFE was taken as control. To all reaction systems 200 μg/ml of kanamycin was added to avoid contamination.

After overnight incubation at 28°C ± 2°C the reactions were stopped by acidifying to pH 2.0 with 6N HCl, extracted twice with diethylether, and the organic layer were subjected to HPLC analysis.
Enzyme Studies:

2-chlorobenzoate-2-hydroxylase (EC. 1.14.13 x)

The enzyme was assayed spectrophotometrically as described by Shailubhai et al. (5) for benzoate 4-hydroxylase. The rate of the reaction was monitored by observing the NADH oxidation at 340 nm; in a reaction mixture containing (in μmoles): Tris-HCl buffer (pH 7.0), 100; NADH, 0.5; FAD, 0.02; 2-chlorobenzoate, 500; and an appropriate amount of the enzyme protein to a final volume of 3 ml. One unit of enzyme was defined as that which catalyses the disappearance of 1.0 μmole of NADH per min. at 28 ± 2°C.

Salicylate Hydrolyase (EC 1.14.13.1)

The enzyme was assayed by the method described by Yamamoto et al. (6). The rate of the reaction was monitored by observing the NADH oxidation at 340 nm. The reaction mixture contained (in μmoles): FAD, 0.02; NADH, 0.5; Salicylate, 500; and Tris-HCl buffer (pH 7.0) 100; and an appropriate amount of the enzyme protein to a final volume of 3 ml. One unit of enzyme was defined as that which catalyses the disappearance of 1.0 μmole of NADH per min. at 28 ± 2°C.

Catechol 1,2-dioxygenase (EC 1.13.11.1)

The enzyme activity was assayed by the method of Sistrom and Stanier (7). The product cis cis muconate is measured spectrophotometrically at 260 nm. The reaction mixture contained (μmoles): catechol, 0.3; phosphate buffer (pH 7.0), 200; and an appropriate amount of the enzyme protein in a total volume of 3.0 ml. One unit was defined as the amount of enzyme required to bring about a change by 0.1 OD/min. at 260 nm at 28 ± 2°C.

Catechol 2,3-dioxygenase (EC 1.13.11.2)

The enzyme was assayed by the method of Nozaki (8). Activity was measured by following the formation of 2-hydroxymuconic-ε-semialdehyde at 375 nm. The reaction mixture contained (in μmoles): phosphate buffer (pH
7.0), 200; catechol, 200; and an appropriate amount of enzyme protein in a final volume of 3 ml. One unit was defined as the amount of enzyme required to bring about a change of 0.1 OD/min at 28 ± 2°C.

**Succinate Dehydrogenase (EC 1.3.99.1)**

The enzyme was assayed by the method of Arrigoni and Singer (9). The reaction mixture contained (in μmoles): phosphate buffer (pH 7.0); 100; KCN, 3.0; 2,6-dichlorophenol indophenol blue, 0.04; and an appropriate aliquot of the enzyme protein, in a total volume of 2.8 ml. 50 μmoles of succinate and 0.05 ml of 2% phenazinmethosulfate was added and the decrease in OD at 600 nm was determined. One unit of the enzyme activity was defined as the amount of enzyme which causes a decrease of 0.01 OD at 600 nm per min. at 28 ± 2°C.

**Purification of Catechol 1,2-dioxygenase:**

The CFE was prepared as described earlier and was subjected to ammonium sulphate fractionation (25-50% w/v saturation). The precipitates were centrifuged at 18,000 x g for 20 min, and were dissolved in 20 mM Tris-HCl buffer pH 8.0 containing 2 mM mercaptoethanol. These were then dialysed against the same buffer overnight at 10°C. After dialysis, the enzyme preparation was loaded onto a Sephadex G-150 column, having a bed volume of 70 ml and a void volume of 32 ml (determined by the exclusion of blue dextran). The flow rate was adjusted to 0.3 ml/min and 3 ml fractions were collected, using a TOYO fraction collector SF-100 (Japan).

**Estimation of β-ketoadipate:**

β-ketoadipate was determined by using Rothera's colour reaction (10). 0.8 gm of (NH₄)₂SO₄ was added to 0.1 ml of the reaction system followed by the addition of 0.2 ml of 1% (w/v) nitroprusside and 0.5 ml of liquor ammonia. The colour obtained was compared to that of acetone control and read at 545 nm.
Protein Estimation

Protein from cell-free extract was estimated by the method of Bradford (11).

Conjugation:

Conjugation was performed as described by Knackmuss et al. (12). Pseudomonas spp. B13 was a gift from Dr. H. J. Knackmuss (Stuttgart, West Germany). Donor and recipient cultures were grown for 24 hrs at 28 ± 2°C. The bacteria were suspended in 50 mM phosphate buffer pH 7.4 to 5 x 10^8 cells/ml. Portions of each culture were mixed so that the ratio of donor and recipient was 2:1 unless otherwise stated. This mixture was filtered through 0.2 μm millipore filter (Sartorius Co.). The filter, after being placed on the surface of luria plate was incubated at 28 ± 2°C for 12-16 hrs, and then the filter was placed in 15 ml of 20 mM phosphate buffer pH 7.0 and vigorously shaken to resuspend the cells. The resulting suspension was plated on 3-chlorobenzoate plates (0.1% w/v) containing 200 μg/ml of streptomycin and 10 μg/ml of tetracycline or 2-chlorobenzoate (0.2% w/v) plates containing 200 μg/ml of streptomycin. The conjugational frequency was defined as the number of exconjugants per recipient cell population.

Transposon mutagenesis was performed similarly by conjugational transfer of Tn5 suicide vector. The antibiotic concentrations used in the study are as follows, unless otherwise stated.

Chloramphenicol (Chl) 100 μg/ml; Tetracycline (Tc) 25 μg/ml; Carbenicillin (Cb) 100 μg/ml; Kanamycin (Km) 100 μg/ml; Streptomycin (Sm) 200 μg/ml and Rifampicin (rif) 150 μg/ml.

Plasmid Isolation:

Plasmids were isolated by 'alkaline lysis' method as described by Keiser (13).
Solutions :

a) Alkaline SDS : 0.3 M NaOH; 2% SDS
b) Phenol/chloroform : 5 gms phenol, 5 ml chloroform, 1 ml water and 5 mg 8-hydroxyquinoline.
c) Lysozyme solution: 2 mg/ml lysozyme in 0.3 M sucrose, 25 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 50 µg/ml RNAse (preheated 90°C for 10 mins).

Buffers :

Tris-EDTA : 10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0)

Agarose gel electrophoresis buffer :

Tris-acetate EDTA : 40 mM Tris-acetate; 1 mM EDTA (pH 8.2).

Method :

The bacterial pellets were suspended in 500 µl of lysozyme solution by gentle mixing. It was then incubated at 37°C for 30 mins. Subsequently, 250 µl of alkaline SDS solution was added, contents were uniformly mixed and incubated at 55°C for 30 mins. To this 80 µl of phenol/chloroform solution was added, it was then thoroughly mixed and centrifuged in an Eppendorf Centrifuge till two phases were separated. The aqueous phase was removed and loaded in the well of 0.7% (w/v) agarose gel using loading buffer (0.25% bromophenol blue in 50% glycerol). Submerged gels were run at a constant voltage of 60 V. Gels were stained with ethidium bromide (0.5 µgm/ml) for 10-15 mins after completion of run and were observed in a Fotodyne photosystem 1000 transilluminator (UV 310 nm). Gels were photographed using a polaroid MP-4 camera attached to the photosystem 1000.

Caesium Chloride Density Gradient Centrifugation :

The plasmid was extracted from 3 litres of exponentially growing cells as described earlier. To 10 ml of the lysate solid caesium chloride was added so as to obtain a final concentration of 0.8 gm/ml to 1.5 gm/ml (w/v) of caesium chloride. To this 0.8 ml of a solution of ethidium bromide (10 mg/ml
in water) was added. The contents were transferred to a 12.5 ml polyallomer ultracentrifuge tubes and the tubes were centrifuged at 250,000 x g in a fixed angle rotor TFT 65.38 in a Kontron TGA ultracentrifuge for 36 hrs. at 20°C. Two bands of DNA were visible in UV light which were extracted separately using sterile syringes. To the extracted bands equal volume of isomylalcohol was added and mixed vigorously to remove ethidium bromide. The extraction was repeated 4-6 times until all the pink colour disappeared from the aqueous solution. The aqueous phase was dialysed against several transfer of Tris-EDTA buffer pH 8.0.

**Digestion with Restriction Endonucleases:**

EcoRI and Hind III were purchased from Boehringer Knoll (W. Germany). Digestions were carried out overnight at 37°C in the following buffers. EcoRI buffer (10 mM MgCl$_2$, 100 mM NaCl, 50 mM Tris-HCl pH 7.5 and 1 mM dithiothreitol) and Hind III buffer (10 mM MgCl$_2$, 50 mM NaCl, 10 mM Tris-HCl pH 7.5 and 1 mM dithiothreitol). After overnight incubation, the fragments were analysed by agarose gel electrophoresis.
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Cold Spring Harbor Laboratory, New York.