CHAPTER II
MATERIALS AND METHODS
II.1. MATERIALS

CHEMICALS

Cypermethrin and fenvalerate were generous gifts from the Rallis. Agrochemicals India Ltd. Mumbi, India. 3-Phenoxybenzoic acid was obtained from Sigma-Aldrich; USA. Polyurethane foam, nylon meshes were purchased from local market. The following chemicals were purchased from Sigma Chemicals Co. St. Louis, USA; Sodium alginate, nutrient agar, agar-agar, sodium dodecyl sulphate (SDS), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, gentisic acid, catechol, 4-aminobenzoic acid, 2,3-dihydroxybenzoic acid, 4-chlorobenzoic acid, 2,6-dichlorophenol indophenol, dithiothreitol (DTT), glutathione (GSH), calcium chloride, 2-mercaptoethanol, iodoacetate, protamine sulphate, bovine serum albumin (BSA), NAD⁺, NADP⁺, NADPH, FAD, acrylamide, N, N'-methylene-bis-acrylamide, N, N, N', N'-tetramethylene ethylene-diamine (TEMED), DEAE-Cellulose, Sephadex G-150, α,α'-dipyridyl and α-phenanthroline. All other chemicals used were of analytical grade available commercially.
ORGANISMS

Microorganisms isolated from the contaminated soil samples by enrichment cultures were used in the present studies.

II. 2. METHODS

II.2.1. Composition of the growth medium

The mineral salts medium described by Seubert (1960), having the following composition was used for the growth of bacterial cultures.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>6.30g</td>
</tr>
<tr>
<td>$KH_2PO_4$</td>
<td>1.82g</td>
</tr>
<tr>
<td>$NH_4NO_3$</td>
<td>1.00g</td>
</tr>
<tr>
<td>$MgSO_4 \cdot 7H_2O$</td>
<td>0.20g</td>
</tr>
<tr>
<td>$CaCl_2 \cdot 2H_2O$</td>
<td>0.10g</td>
</tr>
<tr>
<td>$FeSO_4 \cdot 7H_2O$</td>
<td>0.10g</td>
</tr>
<tr>
<td>$Na_2MoO_4 \cdot 2H_2O$</td>
<td>0.06g</td>
</tr>
<tr>
<td>$MnSO_4 \cdot H_2O$</td>
<td>0.06g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.00 litre</td>
</tr>
</tbody>
</table>

The medium was boiled, filtered and the pH was adjusted to 7.0. It was then dispensed in 100 ml quantities in 500 ml Erlenmeyer flasks and sterilized by autoclaving for 20 min. at 15psi. Substrates were added to the autoclaved medium just before inoculation. Solid media contained 1.5% agar with mineral salts medium.
II.2.2. Growth conditions

The isolated bacterium was routinely grown in the mineral salts medium supplemented with aromatic substrates as sole source of carbon and energy. The 24 h grown starter culture (2 ml) was inoculated into each flask and incubated aerobically in the dark at room temperature (around 28°C) on a rotary shaker at 150 rpm. After the desired time of growth, the cells were harvested by centrifugation at 10,000-x g for 20 min. The cells were washed twice with 0.05 M phosphate buffer, pH 7.0 and used directly for metabolic and enzymatic studies. Growth of the organism was measured turbidometrically by monitoring the O.D at 660 nm. Cell growth was also determined by weighing the dried cells.

II.2.3. Maintenance of the organisms

The organism was maintained on the substrate- mineral salts- agar slants at 4°C, subculturing once a month. The cultures were also maintained on nutrient agar slants covered with layer of sterile liquid paraffin at room temperature for several months.

II.2.4. Replacement culture experiments

The replacement culture experiments were carried out with bacterial cells washed with sterile 0.05 M phosphate buffer, pH 7.0 and resuspended in the same buffer containing 0.1% of different aromatic substrates. The replacement cultures were incubated on a rotary shaker at room temperature. Cell-free controls to account for biological transformations were included with all incubations. The samples were taken from the cultures at regular intervals and analyzed for residual substrates and metabolites.
11.2.5. Mode of ring cleavage of catechol and protocatechuate

The isolated organisms were tested for mode of ring cleavage of catechol or protocatechuate by “ortho” or “meta” pathway as described by Stanier et al. (1966). Freshly grown cells (0.2 g) were suspended in 4 ml of 0.02 M Tris-HCl buffer (pH 8.0) with 4 ml of 0.01M catechol or protocatechuate. The tubes were shaken and the development of bright yellow colouration within a few minutes indicates the meta-cleavage of the substrate. If no colouration appears the mixture was further shaken for 2 h and tested for β-ketoadipate by the Rothera reaction (Rothera, 1908). About 1g (NH₄)₂SO₄ crystals were added followed by one drop of 1% sodium nitroprusside solution and then by about 0.5 ml of 0.880 sp. gr. ammonia. After mixing, the development of a deep violet colouration indicates the ortho-cleavage of the substrate.

11.2.6. Isolation and identification of metabolites

The metabolites were isolated from the growth and replacement media. After various incubation periods, the content of a whole culture was filtered through a Whatman No. 40 paper and centrifuged at 8,000-x g for 10 min. The supernatant was acidified to pH 2.0 with 2N HCl and extracted twice with diethylether or ethylacetate. The combined ether or ethylacetate layers were subjected to the fractionation procedure as shown in Fig II.1. The acidic, neutral and phenolic fractions thus obtained were dried over anhydrous Na₂SO₄ and evaporated in vacuo. The residues were analyzed for metabolites by thin layer chromatography on silica gel G plates using the following solvent systems:

A) Benzene-dioxane-acetic acid (74: 2: 2, v/v)

B) Toluene-diethylether-acetic acid (75: 25: 1, v/v)
Fig II.1. Fractionation of metabolites from culture filtrates.
C) Chloroform-acetic acid
D) Toluene-dioxane-acetic acid
E) Chloroform-acetic acid

The metabolites were visualized under UV light (at 254 nm) or by exposure to iodine vapours and also by spraying with 1% FeCl₃-K₃Fe (CN)₆ solution in water. Phenolic compounds were detected by spraying with Folin-Ciocalteu's phenol reagent and by their characteristic colour reaction with diazotized p-nitroaniline or with Gibbs reagent [2% (w/v) 2,6-dichloroquinone-4-chloroimide in methanol]. Ortho-dihydroxy compounds were detected by spraying with Arnow's reagent (Arnow, 1937). Aldehydes were detected by spraying with solution of 2,4-dinitrophenylhydrazine (0.1%) in 2 M HCl.

The metabolites were isolated by preparative TLC using suitable solvents and identified by their physico-chemical properties:

a) Comparison of their R₉ values in different solvent systems with those of the authentic compounds.

b) Colour reactions.

c) Melting points/ Boiling points.

d) UV-visible spectra.

e) HPLC.

f) Infrared spectra.

g) NMR spectra.

h) Mass spectra.
II.2.7. Oxygen uptake studies

The oxygen uptake by whole cells was measured in an Oxygraph fitted with Clark oxygen electrode (Hansatech, Germany). The cells were harvested in the early logarithmic phase by centrifugation at 12,000-x g for 20 min. and washed twice with 0.05 M phosphate buffer (pH 7.5). The reaction mixture (1 ml) contained 0.8 ml of 0.05 M phosphate buffer (pH 7.5), 0.1 ml of substrate (1 μmol) and washed cells (2 mg dry wt.). Oxygen uptake rates are expressed as nmol of O$_2$ consumed min$^{-1}$-mg$^{-1}$ of dry cells. The values were corrected for endogenous respiration.

II.2.8. Preparation of Cell-free extracts

Cell-free extracts were prepared from the washed cells suspended in three volumes of 0.05 M phosphate buffer, pH 7.5 by sonication for 5 min. using a ultrasonic processor, model XL 2010 and then centrifugation at 15,000-x g for 40 min at 4°C. The clear supernatant was used as crude extract for enzyme assays.

II.2.9. Enzyme assays

The following enzymes were assayed in the whole cells or cell-free extracts as described below.

a) p-Cresol methylhydroxylase

The p-cresol-dependent reduction of 2,6-dichlorophenol indophenol was followed spectrophotometrically at 600 nm ($\varepsilon_{600nm} = 22,000$ M$^{-1}$ x cm$^{-1}$). The assay mixture contained 0.05 M phosphate buffer, pH 7.5 (2.8 ml), 0.1 mM
dichlorophenol indophenol (20 μl), 1mM phenazine methosulfate (10 μl) and whole cells (0.1 ml). The reaction was started by adding 0.05 M p-cresol (0.1 ml). (Rudolphi et al., 1991).

b) p-Cresol-2-hydroxylase

p-Cresol-2-hydroxylase was assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The reaction mixture contained 0.15 mM NADPH (0.1 ml), 1.0 mM p-cresol (0.1 ml) and cell free extract (0.1 ml). Assay was performed at 30 °C in 42 mM sodium phosphate buffer, pH 7.1 (2.7 ml). (Jones et al., 1993).

c) 4-Hydroxybenzyl alcohol dehydrogenase

4-Hydroxybenzyl alcohol dehydrogenase was assayed spectrophotometrically by following the formation of NADH at 340 nm. Reaction mixture contained 50 mM glycine/NaOH buffer, pH 9.6 (2.7 ml), 0.4 μmol of NAD+ (0.1 ml), 4 μmol of substrate (0.1 ml) and cell free extract (0.1 ml). This enzyme was assayed at 340 nm and a molar absorption coefficient of 2.86 x 10^3 litre mol^-1 cm^-1 was used for NADH. (Keat and Hopper, 1978).

d) 4-Hydroxybenzaldehyde dehydrogenase

4-Hydroxybenzaldehyde dehydrogenase was assayed spectrophotometrically by following formation of NADH at 340 nm. Reaction mixture contained 0.2 mM NAD+ (0.1 ml), 1.0 mM substrate (0.1 ml), cell free extract (0.1 ml) and 42 mM sodium-potassium phosphate buffer, pH 7.1 (2.7 ml) at 30 °C.
The enzyme was assayed by increase in absorbance at 340 nm (Jones et al., 1993).

e) 4-Hydroxybenzoate-1-hydroxylase

4-Hydroxybenzoate-1-hydroxylase was assayed spectrophotometrically at 30 °C by measuring the rate of decrease in absorbance at 340 nm, due to the substrate dependence oxidation of NADPH. The reaction mixture contained 30 μM NADPH (0.1 ml), 50 mM potassium phosphate buffer, pH 7.5 (2.7 ml), 0.2 M substrate (0.1 ml) and cell free extract (0.1 ml). (Suarez et al., 1991).

f) Gentisate-1,2-dioxygenase

Gentisate-1,2-dioxygenase activity was assayed spectrophotometrically by measuring increase in absorbance at 334 nm, due to the formation of maleylpyruvate. The reaction mixture contained 1.6 mM gentisic acid (0.1 ml), 20 mM Tris-HCl buffer, pH 8.0 (2.8 ml) and cell free extract (0.1 ml). The molar extinction coefficient used (Δε 330nm was 10.3 x 10^3 M⁻¹ cm⁻¹). (Suarez et al., 1996).

g) 4-Hydroxybenzoate-3-hydroxylase

4-Hydroxybenzoate-3-hydroxylase activity was assayed spectrophotometrically by increase in absorbance at 340 nm, due to the formation of NADH. The assay system contained 0.2 mM NAD⁺(0.1 ml), 1.0 mM substrate (0.1 ml), cell free extract (0.1 ml) and 50 mM potassium phosphate buffer, pH 7.5 (2.7 ml). (Karegoudar et al., 1999).
h) Cypermethrin hydrolysing esterase

Cypermethrin hydrolysing esterase activity was assayed spectrophotometrically by decrease in absorbance at 250 nm, due to the disappearance of substrate. The reaction mixture contained 1.0 mM substrate (0.1 ml) in methanol, 50 mM phosphate buffer, pH 7.5 (2.8 ml) and cell free extract (0.1 ml).

i) 3-Phenoxybenzaldehyde dehydrogenase

3-Phenoxybenzaldehyde dehydrogenase activity was assayed spectrophotometrically by increase in absorbance at 340 nm, due to the formation of NADH. The assay system contained 0.2 mM NAD+(0.1 ml), 1.0 mM substrate (0.1 ml), cell free extract (0.1 ml) and 50 mM potassium phosphate buffer, pH 7.5 (2.7 ml).

j) 3-Phenoxybenzoate-4-hydroxylase

3-Phenoxybenzoate-4-hydroxylase was assayed spectrophotometrically at 30 °C by measuring the rate of decrease in absorbance at 340 nm, due to the substrate dependence oxidation of NADPH. The reaction mixture contained 30 μM NADPH (0.1 ml), 50 mM potassium phosphate buffer, pH 7.5 (2.7 ml), 0.2 M substrate (0.1 ml) and cell free extract (0.1 ml).

k) 3-Phenoxybenzoate dioxygenase

3-Phenoxybenzoate dioxygenase activity was assayed spectrophotometrically at 30 °C by measuring the increase in absorbance at 295 nm. The assay system contained 0.5 mM of substrate (0.1 ml), 50 mM phosphate buffer, pH 7.5 (2.8 ml) and cell free extract (0.1 ml).
l) Phenol hydroxylase

Phenol hydroxylase activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm, due to the oxidation of NADPH. The reaction mixture contained 42 mM phosphate buffer, pH 7.1 (2.7 ml), 0.15 mM NADPH (0.1 ml), 0.5 mM phenol (0.1 ml) and cell free extract (0.1 ml) (Neujahr and Gaal, 1973).

m) Protocatechuate -2,3-dioxygenase

Protocatechuate-2,3-dioxygenase activity was assayed spectrophotometrically by observing accumulation of 2-hydroxymuconic semialdehyde at 375 nm. The total assay mixture contained 0.1M phosphate buffer, pH 7.2 (2.8 ml), 10 mM protocatechuic acid (0.1 ml) and cell free extract (0.1 ml). (Crawford, 1975).

n) Protocatechuate -3,4-dioxygenase

Protocatechuate-3,4-dioxygenase activity was assayed spectrophotometrically by measuring the decrease in absorbance at 290 nm, due to the disappearance of protocatechuic acid. The reaction mixture contained 0.05 M phosphate buffer, pH 7.0 (2.8 ml), 10 mM protocatechuic acid (0.1 ml) and cell free extract (0.1 ml). The reaction was initiated by the addition of the substrate. (Fujisawa and Hayaishi, 1968).

o) Protocatechuate -4,5-dioxygenase

Protocatechuate-4,5-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 410 nm, due to the formation of 4-carboxy-2-hydroxymuconic semialdehyde (CHMSA) from protocatechuate. The
reaction mixture contained 0.05 M Tris – HCl buffer, pH 7.4 (2.8 ml), 10 mM protocatechuic acid (0.1 ml) and cell free extract (0.1 ml). (Dagley et al., 1968).

p) Catechol-1,2-dioxygenase

Catechol-1,2-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 260 nm due to the formation of cis,cis-muconic acid from catechol. The reaction mixture contained 50 mM phosphate buffer, pH 7.0 (2.8 ml), 1 mM catechol (0.1 ml) and 0.1 ml of cell free extract (0.1 ml). (Hayaishi et al., 1957).

q) Catechol-2,3-dioxygenase

Catechol-2,3-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 375 nm, due to the formation of 2-hydroxymuconic semialdehyde from catechol. The reaction mixture contained 10 mM phosphate buffer, pH 7.0 (2.8 ml), cell free extract (0.1 ml) and 10 mM catechol (0.1 ml). (Kim et al., 1992).

II.2.10. Protein estimation

The protein content of various enzyme preparations were estimated by the method of Lowry et al (1951) using bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount of the enzyme, which catalyzes the consumption of 1 μmol of substrate or formation of 1 μmol of product per min under the specified standard conditions. Specific activities were expressed as units per mg protein.
II.2.11. Polyacrylamide gel electrophoresis (PAGE)

Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis (1964). The separating and stacking gel used were 7.5% and 5% respectively. Tris-glycine buffer, pH 7.2 (0.05 M) was used as the electrode tray buffer and bromophenol blue (0.02%) as the tracking dye. Electrophoresis was carried out by applying a current of 3mA per tube and was continued till the dye band reached the lower end of the gel. After electrophoresis, the gels were stained with 0.25% coomassie brilliant blue-R in methanol-acetic acid (45: 5, v/v). The gel was subsequently destained with methanol-acetic acid-water (40: 5: 55, v/v).

II.2.12. Analytical methods

Metabolites were analyzed by reversed-phase HPLC with a 5μ spherisorb-ODS (C18) column (25cm x 4.6mm) and acetonitrile-phosphate buffer (50 mM phosphate buffer, pH 7.0) as the mobile phase. The flow rate was 1ml/min. The peaks were detected at 280nm. The mass spectra were recorded with a mass spectrometer Jeol, MS-DS 303 operated at -70eV. UV-visible absorbance spectra were recorded with ‘Hitachi’ 150-20 spectrophotometer. Infrared spectra of the compounds were obtained on KBr pellets by using ‘Nicolet impact 410’ FT-IR spectrometer. The proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Bruker Amx-300, 300MHz spectrometer using TMS as internal standard.