CHAPTER II

MATERIALS AND METHODS
2.1 MATERIALS

All chemicals used for the preparation of the media for the propagation of the microbial cultures were of reagent grade and those used for the metabolic investigations were of analytical grade. The various compounds used in these studies were obtained from the different commercial firms viz. B.D.H., Sigma and Aldrich. Dimethylterephthalate was obtained from SISCO, India, terephthalate and p-nitro-benzyl bromide were obtained from Fluka, Buchs, Switzerland. Phthalic acid, homophthalic acid, benzoic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, protocatechuic acid and catechol were obtained from Aldrich, USA. Gentisic acid nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cytochrome-c and reduced glutathione were procured from Sigma, U.S.A. Monomethylterephthalate and p-nitrobenzyl ester derivatives were synthesised in the laboratory.
2.2 EXPERIMENTAL METHODS

A bacterial strain was isolated from garden soil by dimethlterephthalate enrichment culture technique and *Sclerotium rolfsii* was obtained from the University of Agricultural Sciences, Dharwad.

2.2.1 Maintenance and propagation of cultures

The microorganisms were maintained and propagated on nutrient agar and substrate-mineral salt agar media. The microorganisms were grown on nutrient agar medium of the following composition (Lapage and Shelton, 1970).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0 gm</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>100 ml</strong></td>
</tr>
</tbody>
</table>

First four constituents were dissolved in distilled water and the pH was adjusted to 7.2 with 4M sodium hydroxide. The final volume was made up to 100 ml with distilled water. Then agar was added to the solution and
the mixture was steamed for one hour. For the preparation of slants, an aliquot of 6-7 ml of mixture was taken into a 18x150 mm corning glass test tube, sterilised at 15 p.s.i. (120°C) for 20 mins and were slanted. The slants were resteeamed for one hour to destroy any germinated spores of contaminants and were reslanted again. These slants were inoculated from the broth or from the stock cultures and were then incubated at 37°C for 24 hrs. These cultures were kept viable upto moderate periods by maintaining at 5°C. Under certain conditions the cultures can be propagated even on glucose-mineral salt agar slants. It was also possible to maintain and keep the cultures viable at room temperature for long periods by covering the growth surface of the nutrient agar slants with a layer of sterile liquid paraffin.

2.2.1.1 Mineral salt medium

The mineral salts medium as described by Seubert (1960) having following composition was used for the growth of the bacterium:
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>6.30 gm</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.82 gm</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>1.00 gm</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.10 gm</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.10 gm</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.10 gm</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.60 mg</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.60 mg</td>
</tr>
</tbody>
</table>

Total volume 1 litre

_Sclerotium rolfsii_ was grown on mineral salt medium of the following composition (Byrde et al., 1956).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.00 gm</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>5.00 gm</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>2.50 gm</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>1.00 gm</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>1.00 gm</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>20.00 mg</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>10.00 mg</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>3.00 mg</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>1.50 mg</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>1.00 mg</td>
</tr>
</tbody>
</table>

Total volume 1 litre
All the constituents were dissolved in 1 litre of distilled water. The solution was boiled, filtered and the pH was adjusted to 7.0 using sodium hydroxide solution. Aliquots of 200 ml of this medium were taken in 500 ml Erlenmayer flasks and these were sterilized at 15 p.s.i. for 20 mins. The flasks were then inoculated under the aseptic condition with 5% inoculum and 0.01% substrate was added. These were incubated at room temperature on a rotary shaker for 24 hrs. For routine studies the organisms were maintained by frequent transfers in liquid cultures.

2.2.2 Growth studies

The cells adapted to a specific substrate were used for the growth study of microorganisms. The substrate grown cells were freed from adhering substrate by centrifugation at 5000 rpm for 20 mins at 5°C and washed with 0.05M phosphate buffer (pH 7.0) and resuspended in sterile mineral salt medium. All these operations were performed under the aseptic conditions. Suitable aliquots of 2 ml cell suspension were inoculated into flasks containing each about 0.15-0.20% of various substrates and incubated at 37°C temperature. The growth of the organism was measured turbidometrically by
monitoring the optical density at 660 nm at different incubation periods.

2.2.3 Isolation and identification of metabolites

In order to trace the microbial degradative patterns, the strain was grown on mineral salt medium containing the substrate. The culture was grown on a large scale to facilitate an appreciable accumulation of the metabolites. The metabolites were isolated and identified using various analytical methods after extraction with diethyl ether from the culture broth. At the end of the incubation period, the flasks were pooled together and the whole broth was centrifuged at 5,000 rpm for about 20 mins to remove the cells. The supernatant was then acidified with 4M HCl to pH 2.0 and extracted repeatedly with equal volumes of peroxide free diethylether. The ether extracts were dried over anhydrous sodium sulphate and the ether was evaporated in vacuo. The metabolites from a residue were isolated and purified by chromatography.

The metabolites were purified by preparative thin layer chromatography. It was performed on silica gel plates using the following solvent systems:
(A) Benzene-dioxan-acetic acid (75:2:1 v/v)
(B) Benzene-acetic acid (80:1 v/v)
(C) Benzene-ethylacetate-acetic acid (95:5:2 v/v)

The spots on the chromatogram were visualized by a UV lamp and were detected with iodine vapour or by spraying with 0.025% ethanolic rhodamine-B solution, or by spraying with 0.1% bromocresol green inethyl alcohol.

The compounds eluted by using ethanol from different portions of a chromatogram developed by preparative TLC were further identified by various spectral methods. The ultraviolet spectra of the samples, using ethanol as the solvent, were recorded in a Hitachi UV-visible spectrophotometer model 150-20. The infrared spectra, using KBr pallets of the samples were recorded in a Hitachi infrared spectrophotometer model 270-30. The nuclear magnetic resonance spectra were recorded on JEOL, model Fx 90Q 90MHz FT NMR using DMSO-d_6 as the solvent. Mass spectra were recorded with mass spectrometer model Hololeke Packard GC S985 (70eV) and JEOL, Model JMS-Dx 303 mass spectrometer.
2.2.4 Manometric studies

Measurements of oxygen uptake by cell were carried out manometrically (Umbreit et al. 1972) using Warburg constant volume (25 ml) apparatus. The cell suspension for this study was prepared as follows:

Flasks (500 ml) containing each 200 ml of the mineral salt medium with 0.01% of the substrate were inoculated from 24 hrs culture. The flasks were then incubated at room temperature on a rotary shaker for 24-28 hrs. At the end of the incubation period the cells were harvested by centrifugation (5000 rpm) at 0-5°C for 20 mins. The cells were washed twice with 0.05M phosphate buffer (pH 7.0) and then resuspended in the same buffer to give a final optical density of 1.0 at 660 nm. The dry weight of the cell suspension corresponded to 10-10.5 mg/ml. This cell suspension was used for manometric experiments. 1 ml of the cell suspension and 1.5 ml of 0.05M phosphate buffer (pH 7.0), were placed in the main compartment of each of the Warburg flasks. The different substrates (5-20 μmol) were placed in the side arms of the flasks and 0.2 ml of 20% KOH solution was added to the central wells. Filter paper strips were then soaked in the wells. Distilled water was added to each flask to
adjust the final volume to 3.0 ml. The temperature of the bath was maintained at 30°C and the flasks were shaken at 100 strokes per min. Oxygen uptake was measured after tipping the substrates from a side arm. All the values were corrected for endogenous oxygen consumption.

2.2.5 Enzyme Assays

The key enzymes involved in the metabolic pathway were assayed by using the cell free extract. Fresh cells from the early logarithmic growth phase of the cultures were harvested by centrifugation (5000 rpm) at 0-5°C for 20 mins. The cells were washed twice with 0.1M phosphate buffer (pH 7.0) and were then made into a paste by gradual addition of the phosphate buffer. The cell paste was subjected to sonication (Braunsonic sonicator 1510, USA) at 100W for 10 mins with every two mins of interval to allow for the probe rest and freed of cell debris by centrifugation at 15,000 rpm for 30 mins. The supernatant was collected as the cell free extract (crude enzyme). All these operations were performed at 0-5°C.

For the preparation of cell free extract of fungi, freshly harvested mycelium was washed twice with
distilled water and macerated in a chilled mortar for 15 min with an equal weight of glass powder. It was extracted with 0.025M sodium phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100. The extract was passed through a cheesecloth and centrifuged at 12,000 rpm for 20 min. The supernatent was used as the crude enzyme preparation.

The protein content of the crude enzyme was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

The cell free extract was used for the different enzymatic assays. The activities of the various key enzymes were measured using spectrophotometric, colorimetric and oxygraphic assays.

2.2.5.1 Protocatechuate 3,4-dioxygenase

a) Spectrophotometric assay

The assay system (MacDonald et al. 1954) comprised a total volume of 3.0 ml, containing 1.2 μmol of protocatechuate, 200 μmol of phosphate buffer (pH 7.0), a suitable amount of the crude enzyme and water, in a
cuvette of 1 cm light path. The enzyme activity at 30°C was measured spectrophotometrically by monitoring the decrease in absorbance at 290 nm due to the disappearance of the protocatechuate.

b) Colorimetric assay

The assay system (Nair and Vaidyanathan, 1964) in a total volume of 1.0 ml contained 0.5 μmol of protocatechuate, 100 μmol of phosphate buffer (pH 7.0) and an appropriate amount of crude enzyme. The mixture was incubated at 30°C for 10 mins. A 0.2 ml of 50% trichloroacetic acid solution was added to the mixture to precipitate out the proteins which were separated by centrifugation. Then 1.0 ml of 10% sodium tungstate, 0.5 ml of 0.5N HCl and 1.0 ml of freshly prepared 0.5% sodium nitrite were added to the supernatent. The mixture was allowed to stand for 3 mins to facilitate the reaction. Then, on addition of 2.0 ml of 0.5N NaOH, the solution developed cherry red colour due to the presence of protocatechuate. The enzyme activity was estimated colorimetrically by measuring the absorbance at 540 nm for the sample solution and the control. The assay mixture which was initially contained trichloroacetic acid served as the control.
2.2.5.2 Catechol 1,2-dioxygenase

The assay system (Hegeman, 1966), comprised a total volume of 3.0 ml, containing 1.0 μmol of catechol, 200 μmol of phosphate buffer (pH 7.0), 10 μmol of EDTA and a suitable amount of the crude enzyme, in a cuvette of 1 cm light path. The enzyme activity was measured spectrophotometrically by monitoring the increase in absorbance at 260 nm due to the formation of cis, cis-muconic acid in the reaction mixture.

2.2.5.3 Gentisate 1,2-dioxygenase

The assay system (Crawford, 1975) in a total volume of 3.0 ml contained 0.15 μmol of gentisic acid, 0.1M sodium phosphate buffer (pH 7.4) and an appropriate amount of the crude enzyme in a cuvette of 1 cm light path. The enzyme activity was measured spectrophotometrically by monitoring the increase in absorbance at 334 nm due to the formation of maleylpyruvate.

2.2.5.4 NADH cytochrome-C reductase

The assay system (William and Kamin, 1962) contained
0.05 \mu mol of cytochrome-C and the crude enzyme in 1.1 ml of 50 \mu mol of potassium phosphate buffer (pH 7.7) with 0.001 M sodium cyanide in a cuvette of 1 cm light path. The reaction was initiated by the addition of 10 \mu mol of NADH. The enzyme activity was measured spectrophotometrically by monitoring the change in absorbance at 550 nm due to the reduction of ferricytochrome-C in the presence of NADH.

2.2.5.5 p-Hydroxybenzoate hydroxylase

a) Manometric assay

The assay system (Gibson, 1970) comprised initially a total volume of 2-3 ml in a Warburg flask containing 100 \mu mol of Tris-HCl buffer (pH 8.0), 0.01 \mu mol of FAD, 10 \mu mol of glucose-6-phosphate, 1.4 units of glucose-6-phosphate dehydrogenase, 10 \mu mol of MgCl$_2$, 0.15 \mu mol of NADP$^+$, 1.0 \mu mol of reduced glutathione and the suitable amount of crude enzyme. The central well of the flask contained 0.2 ml of 5N KOH and the side arm contained 0.5 ml of 0.01M p-hydroxybenzoate. The reaction was initiated by tipping the substrate from the side arm. The enzyme activity was measured by monitoring manometrically the oxidation of p-hydroxybenzoate.
b) Oxygraphic assay

The assay system comprised a total volume of 1.2 ml, containing 120 μmol of phosphate buffer (pH 7.5), 0.5 μmol of NADPH and a suitable amount of crude enzyme, in a Gibson oxygraphic cell. The enzyme activity at 30°C was measured by monitoring oxygraphically the oxidation of p-hydroxybenzoate.

2.2.5.6 Oxidative decarboxylase

The assay system (Elmorsi and Hopper, 1977) in a total volume of 1.0 ml contained 0.5 μmol of terephthalate 50 μmol of phosphate buffer (pH 8.0), 0.12 μmol of NADPH and a suitable amount of the crude enzyme in a cuvette of 1 cm light path. The enzyme activity was measured spectrophotometrically at 30°C by monitoring the decrease in absorbance at 340 nm due to the substrate-dependent oxidation of NADPH.

2.2.5.7 Dimethylterephthalate esterase

Dimethylterephthalate esterase activity was assayed by incubating a suitable amount of crude extract prepared by sonication at 100W for 5 min. All the operations were
carried out at 0-5°C. p-Nitrobenzyl monomethylterephthalate was used as a substrate for the assay. The assay mixture contained 5 μmol of p-nitrobenzyl monomethylterephthalate (500 μl), 100 mM phosphate buffer (400 μl, pH 7.0) and crude extract (10-100 μl) in a sealed vial. It was incubated at 30°C for 10 min. The esterase activity was measured spectrophotometrically by monitoring the increase in absorbance of the product, p-nitrobenzyl alcohol at 276 nm after extracting it with chloroform.

2.2.5.8 Lipase assay

The assay system (Tomizuka et al. 1966) contained 5 ml of emulsion (75 ml of 20% aqueous polyvinylalcohol solution and 25 ml of olive oil) and 4 ml of 0.1M phosphate buffer (pH 7.0). After preheating at 37°C for 10 min, 1 ml of crude enzyme was added and the reaction mixture was kept at 37°C without shaking. Then 20 ml solution of equal volumes of acetone and ethanol was added to stop the reaction. The mixture was titrated with 0.05N sodium hydroxide, using phenolphthalein as an indicator. The lipase activity was calculated from the difference between ten and twenty minutes of reaction time.
2.2.6 Mode of ring cleavage of protocatechuate and catechol

An insight into the different metabolic pathways following the fission of the benzene nucleus of an aromatic substrate is gained by the mode of ring-cleavage of the terminal aromatic metabolites such as protocatechuate and catechol (Stainer et al. 1966) using the whole cells and cell free extract by the method of Eaton and Ribbons (1982a).

a) Method for whole cells: Microbial cells of about 1.0 gm wet weight, together with 50 ml of 0.02M Tris-HCl buffer (pH 7.5) and 50 mg of protocatechuate or catechol were incubated in a 250 ml conical flask. If there was no development of an immediate yellow colour, indicative of the meta-cleavage pathway involving the formation of a semialdehyde, the incubation was prolonged for one hr and the cells were separated by centrifugation at 10,000 rpm for about 20 mins. The supernatant was subjected to Rothera's (1908) test to check the presence of $\alpha$-keto adipate, the end product of ortho-cleavage. The supernatant saturated with ammonium sulphate on addition of freshly prepared sodium nitroprusside and 2 ml of concentrated ammonium hydroxide developed a strong violet
colour indicative of the ortho-cleavage pathway, involving the formation of $\beta$-ketoadipate.

b) Method for cell free extract: The cell free extract of about 0.2 ml, together with 0.8 ml of 50 mM KH$_2$PO$_4$-NaOH buffer (pH 6.8) and 50 ml of 20 mM protocatechuic acid were incubated in a 10ml test tube. If there was no formation of a yellow colour indicative of the meta-cleavage, the mixture was further incubated with shaking for about 30 mins at room temperature. After saturating the mixture with solid ammonium sulphate, two drops of aqueous solution of sodium nitroferricyanide were added and concentrated ammonium hydroxide was layered over the mixture. The development of a deep purple colour within 5-10 mins in the upper layer of the mixture was an indicative of the ortho-cleavage pathway.

2.2.7.1 Preparation of monomethylterephthalic acid

Monomethylterephthalic acid was synthesised by refluxing an equimolar amounts of dimethylterephthalate (3.107 gm) and absolute methanol (50 ml) in the presence of potassium hydroxide (1 gm) at 50°C for about 1 hr on a water bath. The reaction mixture was cooled and was then
acidified with HCl. Monomethylterephthalic acid was extracted with solvent ether and was purified by crystallisation in aqueous ethanol. Further, the purity of the compound was checked by UV, IR, NMR and Mass spectra.

2.2.7.2 Preparation of p-nitrobenzyl esters

1 gm of terephthalic acid dissolved in 10 ml of 4% NaOH and 1 gm of p-nitrobenzyl bromide in 10 ml of ethanol were mixed in 100 ml round bottom flask. The solid separated out was dissolved by the addition of sufficient alcohol to produce homogeneity. The reaction mixture was refluxed for about 2 hrs. It was allowed to cool and filtered. The precipitate was washed with alcohol and then with water. It was recrystallized from alcohol. This compound exhibited a single spot on TLC. Melting point was found to be 262°C. The same method was used for the preparation of p-nitrobenzyl monomethylterephthalate. Melting point of this compound was found to be 175°C (Vogel, 1968).