CHAPTER - II

MATERIALS AND METHODS
1.0 Culture Isolation and Identification:
The fungal cultures were isolated from salt pan or sea water in 1983 by streaking the soil samples collected from salt pan or sea water on Sabouraud's agar plates and incubating at 30°C till growth was obtained. *Aspergillus sydowii* and *Cladosporium sphaerospermum* were isolated from salt pan of the Bay of Khambat, a place located about 70 kms from Baroda, India (pH of the soil 7.8 - 8.1). *Asp. variecolor* was isolated from the Arabian sea water (pH 7.6) near Dona Paula, Goa, India. This fungus was predominantly present in sea water sample. The identification of fungal cultures was done with the kind help of Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England. Fungal isolates were maintained on Sabouraud's agar slants supplemented with 2M NaCl.

2.0 Culture Conditions:
2.1 Media:
The composition of Sabouraud's agar used was (in gm/100 ml): glucose, 2.5; peptone, 1.0; NaCl, 0.5 and agar 2.0. The pH was adjusted to 5.5. The composition of the synthetic growth medium was as follows (gm/litre): asparagine, 10; (NH4)2SO4, 3.5; KH2PO4, 10; MgSO4, 2.0; sucrose, 85; CaCl2, 0.075; ZnSO4, 0.010; MnCl2, 0.005; ammonium molybdate, 0.002; Na2B4O7, 0.002 and FeSO4, 0.002. The pH was adjusted to 5.5 (Though the pH of the sea water and salt pan soil were slightly alkaline but optimum growth was observed at pH 5.5). For experiments with *C. sphaerospermum*, asparagine was replaced by yeast extract, 1.0 and casamino acids, 1.0. Control is the medium devoid of NaCl, to which mentioned concentration of NaCl was added for studies. For studies on different carbon source utilization, sucrose was replaced by 0.25M of either glucose, fructose, maltose, xylose, lactose or 1% w/v, of starch and carboxymethyl cellulose. For studies on different nitrogen source utilization, asparagine was replaced by 0.133M (of nitrogen) of either glutamine, (NH4)2SO4, NH4Cl, NH4NO3 or KNO3.

2.2 Condition of Growth:
The cultures were grown in 50 ml liquid medium in 250 ml Erlenmeyer Flasks on a rotary shaker (180 rpm) at 30 ± 2°C for 72 hr unless and otherwise stated. The mycelia were harvested by filtration and were rapidly transferred to -5°C before use.

2.3 Washing of Mycelia:
The mycelia were washed thrice under reduced pressure with 50 mM Tris-HCl buffer (pH 7.2) for enzyme studies. The final washing did not show the presence of NaCl indicating that NaCl was not adhering to mycelia. Mycelia were also observed microscopically for checking bursting of
mycelia before preparation of enzyme extracts, and thus confirming the intactness of mycelia. These results were supported by absence of cytoplasmic enzyme FDP-aldolase in the washed suspension.

2.4 Standardization of Enzyme Extraction Procedure:

Enzyme extraction procedures employed were not only varied to find out suitable conditions for getting maximum activity but other modification in the extraction procedure were also tested like extraction with NaCl. Since, the mold was grown in the presence of 2M NaCl and enzymes from this condition showed higher activities in number of enzymes, the question was therefore asked whether Na\(^+\) accumulated intracellularly, changes (increases) the extractability of enzymes, hence resulting in apparent increase in the enzyme activity. The control (grown in absence of NaCl) mycelia were therefore taken and extracted in the presence of NaCl (0.5 M) in Tris-HCl buffer of pH 7.2. It was observed that there was no significant difference in the activity of isocitrate lyase and malate dehydrogenase but there was decrease in the activity of FDP-aldolase and glucose-6-phosphate dehydrogenase (Table 1). These types of experiments were done after dialysis also, to remove the effect of Na\(^+\) (if temporary modification is done with NaCl) etc. These experiments have finally suggested that Na may not be increasing the extractability of enzymes. The grinding with only Tris-HCl buffer (pH 7.2), without NaCl, was therefore performed and used for subsequent studies.

Table 1: Extraction of enzymes from *Asp. sydowii* in the presence and absence of NaCl.

<table>
<thead>
<tr>
<th>Condition of extraction</th>
<th>Malate dehydrogenase</th>
<th>Glucose-6-phosphate dehydrogenase</th>
<th>Isocitrate lyase</th>
<th>FDP-aldolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>With Tris-HCl buffer pH 7.2 (50 mM)</td>
<td>274.0</td>
<td>236.1</td>
<td>615.0</td>
<td>580.0</td>
</tr>
<tr>
<td>With Tris-HCl buffer pH 7.2 (50 mM) with 0.5M NaCl</td>
<td>290.5</td>
<td>126.5</td>
<td>608.6</td>
<td>488.0</td>
</tr>
</tbody>
</table>

Further, efficiency of procedure for mycelial breakage was checked microscopically after mechanical grinding in the presence of glass powder to ensure proper grinding.
3.0 Subcellular Preparation and Growth Determination:

3.1 Preparation of cell-free extract:

For intracellular enzyme assays, a cell-free extract was prepared in 50 mM Tris-Cl buffer (pH 7.2) by grinding the frozen mycelia with a pestle in a chilled mortar with glass powder. The extract was centrifuged at 15,000 x g for 30 min and the supernatant obtained was used for intracellular enzyme assays.

3.2 Preparation of mycelial ash:

Fungal mycelia were thoroughly washed with distilled water and were exposed to 800°C for 4 hr for ash preparation.

3.3 Dry weight determination:

Wet mycelia were dried at 50°C in oven till constant weight.

3.4 Specific growth rate (μ) determination:

Specific growth rate (μ) was determined from semilogarithmic plots of growth curves and calculated as follows:

\[
\mu = \frac{[2.303 \ (\log x_2 - \log x_1)]}{(t_2 - t_1)}
\]

where \(x_1\) and \(x_2\) are mycelial dry weights at time points \(t_1\) and \(t_2\), respectively.

4.0 Enzyme Assays:

4.1 Invertase: (β-fructofuranoside fructohydrolase EC 3.2.1.26)

Invertase activity was measured by estimating the reducing sugar released according to the method of Bernfeld (1). The test system contained the following in a total reaction mixture of 2.0 ml; 25 μmoles, sucrose; 50 μmoles, acetate buffer (pH 5.5) and an appropriate concentration of enzyme. The incubation was carried out at 37°C for 1 hr. The reaction was terminated by the addition of 1.0 ml of 3,5-dinitrosalicylic acid (DNSA) and boiled for 10 min. After adjusting the volume with D/W to 10 ml, the OD was measured at 540 nm.

A unit was defined as the amount of enzyme required to liberate 1 μmole of reducing sugar per hr at 37°C.
4.2 **Amylase**: (1,4-D glucan maltohydrolase EC 3.2.1.2)

Amylase activity was measured according to the method of Bernfeld (1). The test system contained the following in a total volume of 2.0 ml: starch, 10 mg; phosphate buffer (pH 6.0), 50 μmoles and an appropriate concentration of enzymes. The reducing sugar released was estimated by the method described above.

4.3 **FDP aldolase**: (Fructose 1,6-diphosphate D-glyceraldhyde-3-phosphate lyase EC 4.1.2.13)

Aldolase activity was measured according to the method of Jagannathan et al. (2). The test system contained the following in a total reaction mixture of 3.0 ml: 0.012 M fructose 1,6-diphosphate (sodium salt); hydrazine sulfate, 0.035 M in 0.0003 M EDTA (pH 7.5) and an appropriate amount of enzymes. The change in OD was measured at 240 nm.

A unit of enzyme activity was defined as the amount of enzyme which causes an increase of 0.001 absorbance unit per min at 240 nm under experimental conditions.

4.4 **Malate dehydrogenase**: (L-malate : NAD⁺ oxidoreductase EC 1.1.1.37)

Malate dehydrogenase was assayed according to the method described by Ochoa (3). The test system contained the following in a reaction mixture of 2.0 ml: 0.4 μmoles, oxaloacetate; 0.2 μmoles, NADH; 100 μmoles, Tris-HCl buffer (pH 7.2) and an appropriate amount of enzyme.

A unit of enzyme activity was defined as the amount of enzyme needed to bring about a decrease of 0.001 absorbance unit per min at 340 nm under experimental conditions.

4.5 **Succinate dehydrogenase**: [Succinate: (acceptor) oxidoreductase EC 1.3.99.1]

The method described by King and Howard (4) was used. The test system contained 250 mM, sucrose; 30 mM, Tris-HCl buffer (pH 7.4); 20 mM, phosphate buffer (pH 7.4); 5 mM, magnesium chloride; 1 mM, potassium cyanide; 1 mM, phenazine methosulphate (PMS) and 70 μM, 2,6-dichlorophenol indophenol (DCIP). The rate of reduction of DCIP was followed at 600 nm (EmM = 16.2).
A unit of enzyme activity was defined as the amount of enzyme that causes reduction of one μmole of DCIP per min at 30°C.

4.6 **Isocitrate lyase** : (**threo-D-isocitrate glyoxalate lyase**
EC 4.1.3.1)

Isocitrate lyase was assayed according to the method of Dixon and Kornberg (5). The test system contained the following in a total reaction mixture of 3.0 ml: 5 μmoles, isocitrate; 50 μmoles, Tris-HCl buffer (pH 7.2); 10 μmoles, cysteine-HCl and an appropriate amount of enzyme. The increase in OD was measured at 324 nm.

A unit of enzyme activity was defined as the amount of enzyme which causes an increase of 0.001 absorbance unit per min at 324 nm under experimental conditions.

4.7 **Glucose 6-phosphate dehydrogenase** : (**D-glucose 6-phosphate : NADP⁺ oxidoreductase** EC 1.1.1.49)

Glucose 6-phosphate dehydrogenase was assayed as described by Kornberg and Horecker (6). The test system contained the following in a total volume of 2.0 ml: 6 μmoles, glucose 6-phosphate; 3 μmoles, NADP; 100 μmoles, Tris-HCl buffer (pH 7.2); 10 μmoles, magnesium chloride and an appropriate concentration of enzyme.

A unit was defined as the amount of enzyme required to bring an increase in 0.001 absorbance unit per min at 340 nm under experimental conditions.

4.8 **Malic enzyme** : (**L-malate : NADP⁺ oxidoreductase (decarboxylating)** EC 1.1.1.40)

Malic enzyme was estimated according to the method of Ochoa (7). The test system contained the following in a total volume of 3.0 ml: 20 μmole, L-malate; 1.5 μmole, NADP; 5 μmoles, MnCl₂; 100 μmoles, Tris-HCl buffer (pH 7.2) and an appropriate concentration of enzyme.

A unit was defined as the amount of enzyme required to bring about an increase of 0.001 absorbance unit per min at 340 nm under experimental conditions.
4.9 Glutamate dehydrogenase: [1-glutamate : NAD⁺ oxidoreductase (deaminating) EC 1.4.1.2]

Glutamate dehydrogenase was estimated using the procedure described by Meers and Tempest (8). The following constituents were added in a 2.0 ml test system: 100 μmoles, Tris-HCl buffer (pH 7.2); 15 μmoles, (NH₄)₂ SO₄; 25 μmoles, α-ketoglutarate, 25 μmoles, NADPH and an appropriate amount of enzyme. The decrease in absorbance at 340 nm was followed on a spectrophotometer (Kontron Uvikon 810). Blanks were run wherein NADPH or (NH₄)₂SO₄ was excluded so as to estimate the endogenous reaction due to the presence of these constituents in the enzyme preparation. In cases where blanks gave activity, these values were subtracted from the experimental values.

A unit of enzyme activity was defined as the amount of enzyme needed to bring a decrease in 0.001 absorbance unit per min at 340 nm.

4.10 NADH dehydrogenase:

NADH dehydrogenase was assayed by the method of King and Howard (4).

The reaction system for the enzyme assay was essentially the same as described for succinate dehydrogenase except that PMS and DCIP were substituted with 1 mM potassium ferricyanide (Eₜ₎M = 1).

A unit of enzyme activity was defined as the amount of enzyme that causes reduction of one μmole of K₃Fe(CN)₆ per min at 30°C.

4.11 NADH oxidase:

NADH oxidase activity was checked polarographically. Frozen and thawed preparation of mitochondrial pellet were used for determining the activity of NADH oxidase.

The reaction chamber fitted with Clark-type oxygen electrode contained at 30°C: 50 mM, Tris-HCl buffer (pH 7.4); 2 mM, EDTA; 20 mM, phosphate buffer (pH 7.4); 7 mM, magnesium chloride; 50 mM, sucrose and mitochondrial suspension (0.8-1 mg of protein). 0.5 mM NADH was used as substrate.
A unit of enzyme activity was defined as the amount of enzyme which causes consumption of one ng atom oxygen per min at 30°C.

4.12 Succinooxidase:
Succinooxidase activity from the mitochondrial fraction was checked polarographically. The reaction system was essentially the same as described for NADH oxidase except that succinate (20 mM) was used as a substrate instead of NADH, with proper blank.

A unit of enzyme activity was defined as the amount of enzyme which causes consumption of one ng atom oxygen per min at 30°C.

4.13 Cytochrome oxidase:
Cytochrome oxidase activity from the mitochondrial fraction was checked polarographically. The reaction system was essentially the same as described for NADH oxidase except that ascorbate (3.6 mM) and N,N,N',N'-tetramethyl p-phenylenediamine (TMPD) (18.0 μM) were used as a substrate instead of NADH, with proper blank.

A unit of enzyme activity was defined as the amount of enzyme which causes consumption of one ng atom oxygen per min at 30°C.

5.0 Respiratory Studies:
5.1 Mitochondria isolation:
Mycelia of the cultures grown for 60 hr were harvested, washed with sterile distilled water and filtered through Whatman paper (No. 3). For mycelial disruption (a) the cells were suspended in the chilled vial containing 0.25 M sucrose solution and sonicated with a microtip in, Vibracell ultrasonic processor at setting 3 for 4-5 mins and homogenate was obtained, or (b) cells were kept in a prechilled mortar and pestle with 0.25 M sucrose solution and subjected to gentle grinding and homogenate was obtained.

The homogenate was subjected to differential centrifugation for isolation of mitochondria.
Three different criteria have been followed to ensure the intactness of the mitochondria used in our study:

(a) Stimulation in the rate of respiration by the addition of ADP;
(b) Sensitivity towards cyanide; (c) To check succinate dehydrogenase (SDH) (marker enzyme of mitochondria) in the supernatant of washed mitochondrial pellet.

5.2 Measurements of respiration:

Oxygen consumption was measured polarographically at 30°C with Clark-type oxygen electrode (Gilson Oxygraph model K-ICTC) from a fresh mitochondrial suspension.

The assay system was essentially the same as described for succinoxidase activity (4.12).

For studies with respiratory inhibitors, rotenone, antimycin A and potassium cyanide were added at a concentration of 10, 3 and 500 nmoles/mg protein, respectively. All the solutions were adjusted to pH 7.4 before use.

5.3 Whole cell respiration:

Mycelial pellets obtained for respiratory studies were washed thoroughly and subjected to very gentle mechanical disruption at 4°C. This suspension was used for measuring whole cell respiration instead of mitochondrial suspension.

6.0 Uptake studies:

For uptake studies cells were grown with or without 2M NaCl in the medium, for 60 hrs. Mycelia were harvested and washed with 50 mM phosphate buffer (pH 7.0). Washed cells were resuspended in fresh buffer and incubated in waterbath at 37°C for 30 min. and then immediately transferred to a fresh buffer containing 10 μCi/50 ml of D-glucose-1\textsuperscript{14}C (sp.activity 32.1 mCi/mmole), glutamic acid \textsuperscript{14}C-(U) (sp.activity 195 mCi/mmole) or succinate \textsuperscript{14}C-(U) (sp.activity 16 mCi/mmole). The uptake of radioactive compound by mycelia was monitored for 30 min at 30°C.

The mycelia were separated from radioactive mixture using membrane filters (0.45 μm pore size, Whatman Limited, Maidstone, England) using a Multipore filtration apparatus. The filters were then each washed
with 50 mM phosphate buffer (pH 7.2) containing 10 mM of corresponding non-radioactive substance.

For all the uptake studies the membrane filters plus adhering mycelia were placed in glass scintillation vial, dried under infrared and 10 ml of scintillation fluor solution was added. The fluor solution was composed of 2,5-diphenyloxazole (PPO) 5.0 gm; p-bis (2-(5-phenyloxazolyl) benzene (POPOP) 0.1 gm; naphthalene 120 gm and 1,4-dioxane, 1000 ml. The radioactivity remaining on the filter was determined by using a 1219 Rack Beta Spectral liquid scintillation counter, LKB, Wallac with Quench standard curve fitting.

For uptake studies with various inhibitors, cells were pre-incubated with either potassium cyanide, 2,4-dinitrophenol, p-chloromercury benzoate, EDTA or NaCl for 10 mins and then uptake of the radiolabelled compounds was performed. Intracellular radioactivity was measured as described above.

7.0 Incorporation of Radioactive Compounds in the Protein Fraction:

To determine protein synthesis, the culture was grown in the presence of 10 μCi / 50 ml of DL-leucine-1-14C (sp. activity 60.12 mCi/m mole) as described by Saturani et al. (9). The cells were homogenized and fractionated as mentioned previously. Radioactive
incorporation in TCA precipitable material was determined as described by Saturani et al. (10).

8.0 Isolation and Fractionation of Lipids:

8.1 Lipid extraction:
Lipids were extracted by grinding 72 hr grown mycelia in Sorval omni mixture with 25 ml of chloroform : methanol (2:1 v/v) at 8000 rpm for 10 min. The homogenate was heated at 45°C for 10 min and centrifuged at 5000 g for 5 min (11). Residues were transferred to Soxhlet extractor and extracted with chloroform : methanol (2:1 v/v) continuously for 12 hr. The combined supernatant was evaporated under vacuum. The residue was dissolved in chloroform and washed with 1.0% KCl (12). The chloroform layer was separated and evaporated to constant weight representing the total amount of extractable lipids (13). The residue was redissolved in chloroform and stored at -4°C for further analysis.

8.2 Fractionation of total lipids:
The total lipids were separated into neutral, glyco and phospho lipids by column chromatography (see section 9.2). Neutral lipid fraction was evaporated to dryness under nitrogen and redissolved in chloroform. Further fractionation of neutral lipids was carried out to get monoglycerides, diglycerides, triglycerides, sterol fatty acids and fatty acid methyl ester on preparative TLC (see section 9.1), eluted with chloroform using proper standard and stored at -4°C for further studies. The amount of each lipid fraction was determined gravimetrically.

9.0 Chromatography:

9.1 Thin layer chromatography:
Thin layer silica gel G plates were used for separation and identification of lipids using heptane : ether : acetic acid (80 : 20 :2) as the solvent system and identified by comparison with standards and specific reagents. Lipid spots were detected by specific spray reagent : sulfuric acid and iodine vapour for all lipids (14, 15).
9.2 Column chromatography:

Total lipids were separated into neutral, phospho and glycolipid on silica gel column according to the procedure of Mumma et al. (16) and Jigani et al. (17). Thus, an aliquot of total lipid extract (40 mg/gm of silica gel) taken up in chloroform : methanol (98:2) and separated on silica gel (silica gel for column chromatography less than 0.08 mm, finer than 200 mesh, Acme Synthetic Chemicals, Bombay). Column eluted with 200 ml of chloroform to give neutral lipid, further elution with 150 ml of acetone to get glycolipid and then followed with 150 ml of methanol for phospholipids (polar lipid). The fractions were evaporated to dryness under stream of nitrogen in tared flasks and the amount of each lipid was determined gravimetrically. Lipids were redissolved in chloroform : methanol (1:1) and stored at -4°C.

9.3 Gas liquid chromatography:

Fatty acids were analysed by gas liquid chromatography as methyl ester. The total lipid fraction was saponified by refluxing with 30% KOH (fresh) in ethanol for 30 min (18). The fatty acids were separated from unsaponifiable fraction as follows. The unsaponifiable material was extracted with diethyl ether (1/3 x 3) and the aqueous fraction was acidified with 2N HCl. The fatty acids were extracted from this with diethyl ether (1/3 x 3) and checked using TLC as mentioned previously. Methylation of free fatty acids was carried out in 14% (w/v) boron trifluorine-methanol (Sigma) according to the method of Morrison and Smith (19). The methylated samples were evaporated to dryness under nitrogen, weighed and resuspended into hexane. Analysis of fatty acid ester was done using gas liquid chromatograph (Shimadzu GC-7A) equipped with flame ionisation detector (FID) and printer (C-R 1B chromatopac). A Silar 7C column was operated at 185°C. The detector temperature was held at 250°C. Nitrogen was used as a carrier gas at 40 ml/min. The fatty acids were identified by comparing their retention time with standard fatty acid methyl esters.
10.0 Analysis of Electrolyte and Amino Acids:

10.1 Analysis of electrolytes:
The mycelial ash obtained from control and 2M NaCl grown culture was dissolved individually in 5N HCl and then subjected to electrolyte analysis using GBC-902 double beam atomic absorption spectrophotometer.

10.2 Analysis of amino acid:
Cell-free extract of control and 2M NaCl grown culture was subjected to cold TCA precipitation. Precipitates were collected by centrifugation at 15,000 x g for 20 mins, supernatant was extracted four times with chilled ether to remove excess TCA. (a) To determine intracellular free amino acid pool size, small aliquot of washed supernatant was evaporated to dryness at 50°C under a stream of nitrogen. The residue was taken up in lithium citrate buffer (pH 2.2) for amino acid analysis. (b) To determine amino acid contents of the total proteins, TCA precipitate obtained were washed four times with chilled ether. Finally washed protein pellet was dissolved in measured quantity of Tris-HCl buffer, pH 7.2 (0.05 M). Small amount (≈ 2 mg) of protein was subjected to hydrolysis by putting it in HCl (final concentration 6N) in a sealed vial after flushing the nitrogen under vacuum for 22 hr at 110°C. Hydrolysate was treated as described above. Amino acid analysis was carried out on LKB automatic amino acid analyser alpha plus model 4151 using Ultrapack 8- cation exchange resin-sodium or lithium form with particle size of 8 µm + 0.5 µm.

11.0 Microscopy:

11.1 Light microscopy:
Mycelia were stained with lactophenol blue and were observed under polvvar (Richard-Jung, Geneva) microscope.

11.2 Scanning electron microscope:
Mycelia grown in presence and absence of 2M NaCl for 72 hr were washed several times with sterile distilled water followed by freeze drying. The dehydrated samples were coated with carbon (about 100 A°) followed by gold-palladium (about 200 A°) and
examined in the microscope at an accelerating voltage of 15 kV.

11.3 Transmission electron microscopy:

The cells were fixed in 6% (v/v) glutaraldehyde in phosphate buffer (50 mM pH 7.0) for 2 hr in cold condition. After four washing with phosphate buffer, the samples were post fixed in 2% buffered osmium tetroxide (OsO₄) for 6 hr. After washing with distilled water, the blocks were soaked in 2% aqueous uranyl acetate for 30 min. They were dehydrated in a graded series of acetone and embedded in Spurr's low viscosity epoxy resin (20). Sections of silver-gray range, cut on LKB-microtome, were picked up on formvar coated copper grids, stained with uranyl acetate and lead citrate (21) and observed using JEOL JEM 100SX electron microscope at an accelerating voltage of 80 kV.

12.0 Native Polyacrylamide Gel Electrophoresis:

Protein from cell-free extracts were separated on polyacrylamide gels according to procedure of Davis (22). Tris-glycine buffer was used as electrode buffer. Protein were loaded on the gel at a concentration of 100 μg/slot. The current was adjusted to 3 mA/slot. Bromophenol blue was used as tracking dye.

Glucose 6-phosphate dehydrogenase activity on gels was visualized by the tetrazolium method (23) by incubating gel in a solution containing glucose 6-phosphate (30 mM), NADP (3 mM), nitroblue tetrazolium (0.5 mM), phenazine methosulphate (3 mM), Tris-HCl buffer pH 7.2 (100 mM) and MgCl₂ (10 mM).

Reaction system for localization of malate dehydrogenase activity consisted of Tris-HCl buffer (100 mM, pH 7.2); malic acid (50 mM); NAD (3 mM); nitroblue tetrazolium (0.5 mM) and phenazine methosulfate (3 mM).

For localization of glutamate dehydrogenase the reaction system used was as described above except that malic acid was replaced with glutamate and NAD was replaced by NADP at the same concentration.
Localization of succinate dehydrogenase was carried out by tetrazolium method using succinate (50 mM) as a substrate and NAD (3 mM) as a cofactor in potassium phosphate buffer (100 mM; pH 7.2).

13.0 Extraction and Estimation of Polysomes:

The polysomes from molds were isolated by following the method described by Verma et al. (24). The fungal mycelia (1 gm/4 ml) extracted in TE buffer (contains Tris-HCl pH 8.2; 50 mM : KCl, 500 mM; magnesium acetate, 5 mM) by using homogenizer. The homogenate was centrifuged at 23,000 x g for 10 mins. Pellet was again homogenized in MD buffer (contains Tris-acetate, pH 8.5, 150 mM; magnesium acetate, 10 mM; Triton X-100, 0.5%) and centrifuged at 23,000 x g for 10 min. Combined supernatant was layered over a 3 ml SC buffer (contains Tris-acetate buffer, pH 8.5, 50 mM; sucrose, 1.5 mM; KCl, 20 mM) and centrifuged at 1,05,000 x g for 90 min to get the polysomal pellet. Pellet was suspended in a small volume of polysome buffer (contains Tris-acetate pH 8.5, 50 mM; KCl, 20 mM; magnesium acetate, 10 mM) and centrifuged at 10,000 x g for 10 mins.

The polysomal RNA was analysed on a Uvikon double beam spectrophotometer by observing at 260 nm. Yeast RNA was used as a standard for conversion of the optical density values to the amount of RNA present.

14.0 Chemical Estimations:

14.1 Estimation of protein:

Protein estimation was carried out by the method of Lowry et al. (25) using bovine serum albumin as a standard. For mitochondrial pellet, protein was estimated by the biuret method using bovine serum albumin as a standard.

14.2 Estimation of lipid:

Total lipids were estimated by the method of Bragdon (26).

14.3 Estimation of sugar:

Sugar estimation was carried out according to the method of Ashwell (27).
14.4 Estimation of polyols:

The procedure of Lambert and Neish (28) was suitably modified for polyol estimation. Quantitative conversion of polyols to formaldehyde takes place in presence of periodate. Formaldehyde generated was estimated with 1,8-dihydroxynaphthalene 3,6-sulphonic acid to form a chrome complex.

The reaction system contains 20-80 µgs of polyols in 2.0 ml of distilled water (D/W). 0.1 ml of 10 N H₂SO₄ was added followed by 0.5 ml of 0.1M Na-metaperiodate. Whole system was kept at R.T. for 5 min. Then 10% (w/v) freshly prepared Na-metabisulfite was added (till color disappears) followed by addition of 7.0 ml D/W and 1 ml was removed for estimation with 5 ml chromotropic acid, kept in boiling water bath for 30 min. After cooling, 1 ml of 10% (w/v) thiourea was added and read at 570 nm. A blank set of polyols was run in each set of determination. Glycerol was used as standard.

15.0 Enzymatic Estimation:

15.1 Glycerol estimation:

Glycerol was estimated enzymatically by the method of Wieland (29).

16.0 Reliability of Results:

Virtually all the results presented in the thesis are representative of experiments carried out minimum two to three times yielding similar results.

The procedure for enzyme extraction and assays were optimized for appropriate concentration of enzymes and substrates. The applicability of procedures in fungal systems were checked earlier in our laboratory (30).

Chemicals:

The fine chemicals used in all experiments were obtained from Sigma Chemical Co., Missouri, U.S.A.

All radioactive compounds were obtained from Bhabha Atomic Research Centre, Bombay, India. All other chemicals used during the work were of either analytical grade or reagent grade.