CHAPTER - II

GENERAL EXPERIMENTAL PROCEDURES

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A strain of Aspergillus nidulans obtained from the Division of Mycology and Plant pathology, Indian Agricultural Research Institute, New Delhi, was used in the present investigations. The stock cultures were maintained on potato dextrose agar slopes at 30±2°C, and were preserved at 5°C.

The composition of the medium for the cultivation of the mold was similar to that described by Rao and Modi[1], except that the glucose concentration was kept at 5 gm% and consisted of the following ingredients in gms/litre. D-glucose 50.0; NH_4NO_3, 3.0; KH_2PO_4, 0.3; and $MgSO_4.7H_2O$, 0.25. The pH of the medium was adjusted to 5.6 ~~with 0.1N~~ with 0.1N NaOH before sterilization. A volume of 100ml medium was distributed in 250 ml Erlenmyer flasks and autoclaved at 10 p.s.i. pressure for 15 minutes.

The medium after inoculation with spore suspension prepared from 5-days old culture, was incubated on a rotary shaker (200 rpm) at 30°C. The culture grown in the above conditions was referred to as "normal culture". Biotin deficiency was achieved as per the method of Rao and Modi[1], by the addition of avidin in the growth medium and the culture

was referred to as biotin deficient.

Dry Weight Determinations :

Specified cultures were harvested after 5 days of growth by filtration and washed thoroughly with distilled water. Dry weight was determined after drying mycelia to a constant weight in a constant temperature oven adjusted at 50°C.

Growth of Aspergillus nidulans in Medium Supplemented with Amino acids :

The normal and deficient cultures were grown in the above mentioned medium, and supplemented with different pools of Amino acids containing 10 µgms/ml of each Amino acid. After 5 days of incubation the growth was collected and dry mat weight determined.

Preparation of Cell Free Extracts :

Freshly harvested mycelium was washed with ice-cold distilled water till free from the medium, and gently passed between the filter papers to remove as much water as possible. They were then weighed and used for the preparation of the extracts.

A known weight of the mycelial mass was ground in the cold-room maintained at 0°-4°, with half of its weight of alumina, and 3 volumes of 0.1M phosphate buffer (pH 7.4). The homogenates thus obtained were centrifuged at 15,000 x g for 15 minutes, and the supernatants were referred to as the cell-free extracts. The protein in the cell-free extracts was estimated by the method of Lowry et al[2], using bovine serum albumin as standard.

Avidin 5 units (Nutritional Biochemicals Ohio) was dissolved in 0.05M acetate buffer (pH 5) aseptically.

Extraction of Lipids :

Total lipid was extracted with 10 volumes of chloroform: methanol (2:1 v/v), and was freed from the impurities by the method of Folsch et al[3]. The lipid was estimated gravimetrically after evaporation of solvents to dryness.

Separation and estimation of Phospholipids :

Phospholipids extracted by following the method of Folch et al[3], were chromatographed on thin-layer plates (0.5 mm thick) of Silica gel. H (Merck and Co., Darmstadt, Germany) as per the method of Yano et al[4]. The solvent system used was Chloroform : Methanol : Acetic acid : water

(85:15:10:4 v/v). Phospholipids were visualized by charring the plates at 150°C for 45 minutes, after spraying them with concentrated sulphuric acid diluted to 70% with a saturated solution of $K_2Cr_2O_7$ in water. For tentative identification, phospholipids were detected with the help of 2'-7'-dichloroflourescein.

Estimation of Ammonia :

Ammonia was estimated by the method of Fawcett and Scott[5]. The utilization was calculated from the difference between initial ammonia concentration before incubation, and leftover ammonia after harvesting mycelial mass.

Extraction and Estimation of Amino acids :

Free Amino acid fraction : The method used was similar to that described by Pillai and Srinivasan[6]. The dry mycelium (0.5gms) was ground with 15 ml of 70% ethanol; in water in a glass mortar for about 30 minutes. The grinding was carried out in cold to minimise evaporation. The suspension was centrifuged and the clear supernatant collected in an evaporating dish. The residue was extracted thrice in the same manner. All the clear supernatants were pooled and evaporated to

dryness under low pressure at 45°-50°C. The residue left after evaporation was taken up in 10% iso-propanol in water, centrifuged and supernatant made upto 4 ml. These extracts were used for the analysis of free amino acids.

Bound Amino acid Fraction : The residue left after extraction of the free amino acid fractions were dried to constant weight in a desicator, and 0.2gms of samples hydrolyzed by refluxing with 20 ml of 6N HCl for 24 hours. The hydrolysate was evaporated to dryness on a waterbath, with repeated additions of distilled water to remove the acid present in the solution. The residue obtained was taken up in 10% iso-propanol in water, centrifuged and the supernatant made upto 4 ml. This extract was used for the analysis of total amino acids except tryptophan.

The circular chromatographic technique developed by Giri and Rao[7-8], with the modification introduced by Rao and Wadhawani[9], was used for the separation and identification of amino acids. The quantitative procedure for the estimation of amino acids was essentially the same as that described by Giri et al.[10-11].

Final Procedure :

A circle of 4 cms diameter was drawn from the centre of the filter paper. Solution of known and unknown mixtures of amino acids were applied alternatively along the circumference of the circle using accurately calibrated capillary tube. The adjacent spots were not to coalse. The spots were dried and chromatogram was developed using Patridges solvent (Butanol : Acetic acid : water; 40 : 10 : 50). The paper was air dried and uniformly sprayed with 0.5% ninhydrin in 95% Acetone. After drying in a current of at 65°C for 30 minutes, the zones were cut out and extracted with 4 ml of 75% ethanol containing 0.2 mg of $CuSO_4$, care was taken to avoid contamination of paper by dust or hands. While recovering the chromatogram the distance of the movement of of the solvent was closely controlled. For the purpose of drawing calibration curves of the paper, 9 cms was always kept constant. The optical density was measured at 540 nm. Blank determination was also made with uncoloured area and correction applied in each case.

For tryptophan analysis alkaline hydrolysate was prepared by following the method of Shah and Ramakrishnan[12]. Dry mycelium powder (0.1 gm) was hydrolyzed by refluxing with 20 ml of 14% $Ba(OH)_2$ for 24 hours. The Barium was

removed with a slight excess of 1N H_2SO_4 and the barium sulphate precipitates were thoroughly washed with hot water containing 0.1% (v/v) Acetic acid. The filtrate and washings were combined and concentrated to a small volume, and then evaporated to dryness. The residue was taken up in 10% iso-propanol, centrifuged and the supernatant made up to 20 ml.

The composition of the medium in gm %, for cultivation of culture and production of cellulase using cellulose as carbon source.

Cellulose	0.25 - 1.50
NH_4NO_3	0.30
KH_2PO_4	0.030
$MgSO_4.7H_2O$	0.025
Protease peptone	0.05
Tween 80	0.02 ml

50 ml of medium was distributed in each flask and autoclaved at 15 p.s.i. for 15 minutes. The fermentation was allowed to proceed for 6 days.

Carboxy methyl cellulase or Cx Activity :

This was assayed[13] by mixing 0.5 ml of enzyme preparation with 0.5 ml of 1% carboxymethyl cellulose (B.D.H. Chem. Ltd.)

in 0.05 M Acetate buffer (pH 4.8) and incubating for 60 minutes at 50°C 1 ml of dinitrosalicylic acid reagent was added and the tubes were boiled for 20 minutes, and the reducing sugar produced was measured as glucose. The glucose production was linear to about 1 mg/ml.

Range 100 µgms - 1000 µgms. Final volume was made up with water to 10 ml. The optical density was determined at 540 nm.

One unit of the enzyme is the amount which releases or produces 100 µgms of glucose per hour per ml of system at 50°C.

Preparation of DNSA (Dinitrosalicylic acid) reagent :

1 gm of DNSA powder (B.D.H. Chem. Ltd.) is dissolved in 20 ml of 2N NaOH and the volume is made upto 50 ml with water. Sodium potassium tartarate was added (30 gms), and the volume was made upto 100 ml with distilled water.

Extraction of Melanin :

Bull's procedure[14], was followed for the extraction of Melanin. Melanin was precipitated from the dried mycelia by incubating them in 6NHCl for 24 hours. The precipitates were washed with solvent ether to remove acid. Melanin preparation thus obtained was solubilized in 0.5N NaOH at

60°C for 24 hours. After adjusting the pH to 7.2, the solution was centrifuged at 3000 g for 15 minutes.

The interaction of melanin with cellulase was examined by incubating a known activity of the enzyme with melanin at 37°C for different time intervals, after which the substrate was added to the mixture and the enzyme assayed keeping a suitable control.

Enzyme Assay :

The spectrophotometric measurements were made on Carl-zeiss Spectrophotometer (Model VSU2). Until and unless specified initial reaction rate of enzyme was measured at 30°C, and a unit was defined as the amount of enzyme, which brought about 0.01 change in O.D. per minute under the given experimental conditions. The protein in the cell free extract was estimated as per the method of Lowry et al[2], using serum albumin as standard.

Glutamate dehydrogenase (Ec. 1.4-1.3)

NAD(P)H glutamate dehydrogenase was measured by following the method of Thomulka and Moat[15]. The reaction mixture consisted of 4 µmoles of ammonium sulphate, 1.7 µmoles of reduced NAD or NADP, 100 µmoles of tris- HCl Buffer (pH 7.3),

20 µmoles of α-Ketoglutarate (pH 7.0), and the enzyme preparation in a total volume of 3 ml. Ammonium sulphate was omitted from the blanks and the decrease in absorbance at 340 nm was recorded.

The unit was defined as amount of enzyme that brought about oxidation of 1.0 µmoles of NADPH per minute at 30°C.

Polyacrylamide Gel Electrophoresis of Proteins :

The proteins for electrophoresis were extracted from the mycelium by following the method of Smythe and Anderson[16], and analysed within 12 hours of its extraction.

In general the method of Davis[17], was followed for electrophoretic separation of the proteins. Gels containing 7.5% (w/v) acrylamide and .735gm (w/v) bis-acrylamide were polymerized chemically with potassium per sulphate in an anaerobic condition. The spacer and sample gels were omitted. The density of samples were increased by the addition of sucrose (30% w/v). The sample (20-200 µgms) concentrations were applied on the top of the gels and electrophoresis was carried out in 0.05M tris-glycine buffer (pH 8.4), at 4°C. Total current of 1.5 mA/gel for initial 15 minutes was applied and finally increased to 4 mA/gel; Bromophenol blue was used as a tracer dye.

The proteins in the gel were stained with 0.1% Coomassie blue as per the procedure of Chrambach et al.[18]. The destaining of the gel was performed by repeated washings in 7.5% (v/v) glacial acetic acid.

Glutamate dehydrogenase activity was obtained by the tetrazolium technique of Thurman et al.[19]. Each ml of the incubation mixture contained 100 µmoles of L-glutamic acid (Na Salt), 0.3 mg of NADP, 1 mg of 5-methyl PMS (phenazine methosulphate), 1 mg of nitroblue tetrazolium ml phosphate buffer (0.1 M) of pH 7.2.

Uptake Experiments :

The uptake studies were carried out following the method of Brown and Romano[20]. The cells were grown in basal medium and were harvested by filtration. Mycelia weighing 150 mgs (wet weight) each of, normal, biotin deficient and HEW cultures (cells grown in basal medium containing 1 ml of heat inactivated egg white by autoclaving at 10 p.s.i. for 10 minutes). After washing with cold distilled water the cells were resuspended in 50 mM tris-HCl buffer pH 7.2, unless specified, the cell density was adjusted to 1.5 mgs dry weight/ml. To this, 100 µgms/ml of the desired amino acid was added an aliquot immediately after addition of the amino acid (control zero time) was removed. The samples

were withdrawn at regular time intervals i.e. 5, 10 and 15 min. The residual amino acid contents in the samples were assayed, and the uptake was measured in terms of the disappearance of the amino acid from the medium with respect to time.

Estimation of Amino acids :

The residual amino acid was measured by the ninhydrin method of Spies[21]. Aliquots of the sample were taken and made upto 1 ml with distilled water, and to this, 5 ml of 0.2% ninhydrin in absolute alcohol was added, and the system was incubated at 60°C for 30 minutes in a water bath. The blue colour was measured at 440 nm. Tryptophan was measured by the method of Grahams[22], using 0.5% p-dimethylamino benzaldehyde reagent.

Buffers :

All the buffers and other solutions were prepared using double glass distilled water, as described by Gomri[23].

Medium for Protease Production :

The medium contained all the ingredients of a normal medium mentioned earlier, except that 1 ml of egg white was

added, to the medium, and this flask was referred to as biotin deficient. In the third case 1 ml of heat-inactivated egg white was added, and this was referred to as HEW culture. The protease assay was done from all the above cultures.

Protease Assay :

The assay of proteolytic activity was done routinely by the method of Anson et al.[24]. The reaction system contained 1.5 ml Hemoglobin (Bovine) solution (0.5 mg/ml), dissolved in 0.1M pH 5, The acetate buffer for assaying acid protease activity or the reaction system contained 1.5 ml Hammerstein casein solution (5 mg/ml dissolved in 0.1M pH 7.2 tris-HCl buffer) for assaying neutral protease or, the reaction system contained 1.5 ml Hammerstein casein solution (0.5 mg/ml, dissolved in 0.1M pH 9 phosphate buffer) for assaying alkaline protease, along with an appropriate quantity of enzyme, i.e. 200 µgms protein in a total assay system of 2.5 ml.

The system was incubated routinely at 37°C for one hour and the reaction terminated by 3 ml of 5 percent trichloroacetic acid.

After centrifuging, to 1 ml of supernatant, 5 ml of 0.4N NaOH was added, followed by 1 ml of Folin's reagent.

One unit of enzyme was defined as the amount of enzyme, which caused a change of 0.10 OD at 500 nm/660 nm per minute, under the stated experimental conditions.

Determination of Molecular Weight :

Molecular weight determination was carried out by the method of Andrews[25], by comparing the elution volume of protease with that of the other reference proteins, by gel filtration on Sephadex G-100. The reference proteins used were lipoxidase (Soybean) 102,000, bovine serum albumen (dimer) 135,000, ovaglobulin (eggwhite) 44,000 RNase A (bovine pancrease) 12,700, 5 mg of each protein was determined separately by measuring the protein in each of the fractions by the method of Lowry et al.[2], elution was carried out using 0.05M tris-HCl buffer pH 7.2.

Purification of Intra- and Extracellular Proteases :

Intra-cellular Proteases: A 30% (w/v) crude cell free extract in 0.05M tris-HCl buffer pH 7.2, was made and allowed to stand for sometime, then it was centrifuged at 15,000 x g for 15-20 minutes, and the supernatant was collected discarding the pellet. Sufficient ammonium sulfphate was added to the supernatant fluid to bring the concentration finally to 40% saturation. After 90

minutes the precipitates were collected by centrifuging at 15,000 x g for 15 minutes, and dissolved in an appropriate concentration of 0.05M tris-HCl buffer pH 7.2. To the supernatant fluid ammonium sulphate was added over a short period to bring the final concentration to 90% saturation, and was allowed to stand for additional 2 hours at 0°C. The protein precipitate formed was collected by centrifuging at 15,000 x g for 15 minutes, and then dissolved in appropriate concentration of buffer, and was subjected to Sephadex G-100 column purification, which had previously been calibrated using standard proteins. The elution was performed with tris-HCl buffer pH 7.2, 0.05M. The fractions of 2 ml each were collected and used to assay the enzyme activity, and the protein was determined by the method of Lowry et al.[2].

Extra-cellular Protease Purification: To a known volume of the culture filtrate, chilled Alcohol or Acetone was added in proportions of 1:2 (w/v) under cold conditions to precipitate out some of the proteins. The acetone or alcohol was decantated and the precipitate collected on Whatman No. I. The precipitates were dissolved in 0.05M tris-HCl buffer pH 7.2 and used to assay protease activity.

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