CHAPTER II -

EXPERIMENTAL PROCEDURE
MATERIALS

Microorganisms

Fungal cultures belonging to the group Aspergillus niger (strains NRRL 330 and 337), A. oryzae 499 and A. awamori 3112 were obtained from Northern Regional Research Laboratories (NRRL), Peoria, Illinois. Other strains of A. niger, A. oryzae, A. wentii and Rhizopus species were from the culture collection of the Microbiology Discipline of the Central Food Technological Research Institute, Mysore, India.

BiologicaIs

Yeast extract, malt, peptones, agar, casein hydrolysate were Difco products.

Biochemicals

Bovine serum albumin, trypsin, alcohol dehydrogenase, amylase, pepsin, egg albumin, lysozyme, Blue Dextran-2000, p-chloromercuribenzoate (pGMB), di-isopropylfluorophosphate (DFP), Sephadex G-100, N-bromosuccinimide (NBS), N-ethylmalamide, iodoaceteamide, 2-hydroxy-
benzyl bromide were obtained from the Sigma Chemical Company. Diazyme (glucosamylase) was from the Miles Chemical Laboratory. DNP-leucine, DNP-alanine, DNP-lysine, amylose, amylpectin were from the Biochemical Unit of the V.P. Chest Institute, Delhi. DEAE-cellulose was from Nutritional Biochemical Corporation. Rose Bengal, methylene blue, glucose, maltose, cellulose, lactose, galactose, dextrin, soluble starch, urea, ammonium sulphate, phosphates, sodium nitrate, sodium nitrite, ethylenediaminetetraacetic acid (EDTA), amino acids, were all from the BDH. Acrylamide, bis-acrylamide and other chemicals required for disc gel electrophoresis were from Eastman Organic Chemicals. All other chemicals used were of analytical reagent grade. Alcohol from commercial source was distilled over KOH and used. Corn (Zea mays), jowar (Sorghum vulgare), ragi (Eleusina coracana), navane (Setaria italica), sajjae (Pennisetum typhoides), rice (Oryza sativa), wheat (Triticum vulgare), barley (Hordeum vulgare), corn starch, groundnut protein isolate, were commercial samples. Antifoam silicone compound 21 defoamer was obtained from Metroarc Company, Calcutta.
METHODS

1) Microbiological

Maintenance of culture - All the fungal cultures were maintained on potato dextrose agar slants. They were subcultured every month on the same medium and incubated at 30°C for 3-4 days. After good growth and sporulation, they were kept in refrigerator until use. A set of well-sporulated cultures were filled with sterilized liquid paraffin and kept in a refrigerator. A heavy spore suspension was mixed with sterilized soil and incubated at 30° for 12-15 days and then kept in refrigerator.

Inoculum - For inoculation purposes, the organism was grown on sterilized barley for 72 - 96 hrs at 30° to obtain spores of same age. Then a suspension of spores by addition of sterile water was prepared and the spore suspension used as inoculum. 1.0 ml of spore suspension (3000 - 3500 spores/ml haemocytometer count) was used per flask (250 ml Erlenmeyer flask) containing 50 ml medium. Spore suspension was added to starch-yeast extract medium, grown for 24-48 hrs on a rotary shaker (Emmenevee make, 250 rpm, 4" strokes)
at 30° and the germinated spores used as inoculum in experiments. The medium was adjusted to pH 4.2 by addition of acetic acid before inoculation to prevent amylase production.

All the fermentation studies were carried out by growing the organism in 250 ml Erlenmeyer flasks kept on rotary shaker (Emmenevee) with 4" stroke set to rotate at 250 rpm. Experiments were also carried out using a reciprocating shaker with 2" displacement and 95 strokes per minute. A New Brunswick incubator shaker with facilities for maintenance of temperature was used for controlled temperature growth conditions.

Fermentor - A 20-liter capacity stainless steel fermentor was used to scale up the fermentation. It had facilities for autoclaving the medium, adjustment of agitation, aeration and automatic foam controlling devices. Humidified sterile air was supplied one to two vol/vol/liter of medium, as required.

Medium - Powdered cereals such as corn, barley, jowar, etc. were used as substrates. Known quantities were added to each flask with water to make up the volume, sterilized in steam at 1 kg/cm² and used. For nutritional studies, corn starch (AR grade) medium
was supplemented with inorganic, organic and complex nitrogen sources, phosphates, B-complex vitamins, trace elements were used in separate experiments to evaluate their efforts on growth and production of the enzyme. Details are mentioned in the "Experimental Results".

All the media were sterilized at 1 kg/cm² pressure for 20 minutes, cooled to room temperature and inoculated under asceptic conditions. The normal asceptic and microbiological conditions were followed.

Duplicate flasks were removed periodically as indicated and filtered through cheese cloth for separation of mycelium and centrifuged at 3000 x g for 20 min. at room temperature. The clear centrifugate was used as the enzyme source to estimate the pH, sugars, protein and enzyme activity. Mycelium dry weight was determined by drying at 100°C to constant weight.

2) Enzymatic methods

Glucosamylase activity was determined by the method devised by Fazur and Ando (1959). 4.0 per cent starch (moisture-free basis) (Corn starch, analytical grade) solution was prepared containing 50 ml of sodium acetate buffer (0.05 M) pH 4.5. The reaction mixture
contained 5.0 ml starch solution and 0.2 ml enzyme solution.

Starch reagent was first equilibrated to 60°C in a thermostatically controlled water bath and then the enzyme added; the control received the same amount of boiled enzyme. After 30 minutes' incubation, alkali (1.0 ml of 4N NaOH) was added to inactivate the enzyme activity. Released sugars were estimated by Somogyi (1945) procedure.

One unit of enzyme activity releases one micromole glucose per minute under the test conditions. Specific activity is the number of units per milligram protein.

Amylase activity was determined by the method described by Dunn et al. (1959). To 50.0 ml of 2 per cent starch solution (pH 4.8, 0.1 M citrate-phosphate buffer) at 40°C, 1.0 ml enzyme solution was mixed and incubated at 40°C for 30 minutes. The reaction was stopped by addition of 5.0 ml of 4 N NaOH. Reducing sugars were estimated.

One unit of amylase activity releases one micromole glucose per minute under the test conditions.

Transglucosidase activity was determined by the method described by Pan et al. (1953). To 10.0 ml of 4 per cent maltose
(0.05 M citrate-phosphate buffer, pH 3.5) was added one ml of enzyme (culture brew concentrate) and the mixture incubated at 30°. At intervals of 8, 16, 24 and 48 hrs, 1.0 ml samples were removed and inactivated with 1 N NaOH (1.0 ml). 0.2 - 0.5 ml of the mixture was streaked on paper chromatogram. Presence of isomaltose or panose indicates transglucosidase activity.

A unit of transglucosidase is the amount of enzyme which will synthesize one micromole of panose per minute from maltose under the test conditions.

3) Chemical Methods

Protein - Protein in enzyme sample was assayed using biuret method (Garnwell et al., 1949). 4.0 ml biuret solutions were added to 1.0 ml aliquot of enzyme solution, mixed well and the mixture was allowed to stand for 30 minutes at room temperature. Then the solutions were read at 540 nm using Spectronic 20. Bovine serum albumin was used as a standard.

Partially purified proteins and protein content of column effluents were determined by the method of Warburg and Christian (1941).
Sugar - Reducing sugar formed in enzymatic reactions was estimated by the procedure of Somogyi (1945). Glucose served as the standard. Sugar was also estimated using orcinol sulphuric acid (Neskovic et al., 1972) as well as phenol sulphuric acid procedures (Montgomery, 1961), for analysis of glycoproteins with glucose as the standard.

4) Other procedures

DEAE-cellulose column chromatography - Chromatography on DEAE-cellulose was carried out according to the procedure described by Peterson and Sober (1962).

Determination of molecular weight by gel filtration - Molecular weight of the purified sample was estimated by gel filtration using Sephadex G-100 according to the method described by Whitaker (1963). According to the method, an excellent linear correlation between the logarithm of molecular weight of a protein and the ratio of its elution volume to the void volume was obtained. The ratio of its elution volume to the void volume was independent of protein concentration within a certain range, column
size and ion exchange adsorption but is dependent on temperature for several proteins. Standard proteins (Bovine serum albumin, egg albumin, pepsin, trypsin and alcohol dehydrogenase) were used for calibration and from this the molecular weight of the experimental sample was calculated.

**Disc gel electrophoresis** - Fractionated enzyme sample was analysed by the disc gel electrophoresis following the method of Davis (1964). The mobility of a protein in an electrical field is dependent on the charge and its molecular weight. This technique can separate a mixture of proteins or peptides with samples as small as 10 μg. Separation is achieved in this method by initial concentration of the components of diluted samples into thin disc-like zones.

Disc gel electrophoresis was carried out at pH 8.9 and 4.3 according to the procedure of Davis (1962) and Reisfeld et al. (1965). Details of the runs are described in the experimental part. Gels were stained for one hour with 0.5 per cent amido black in 7 per cent acetic acid and destained for 24 - 48 hrs in 7 per cent acetic acid with several changes.
Specific test for the detection of glucoamylase activity in the gel - Dextrin was incorporated in the gel and after the run, the gels were incubated at 40°, 0.01 M acetate buffer, pH 4.5. After an hour, it was immersed in iodine solution when the gel appears as blue with clear colorless zones where dextrin was hydrolyzed due to enzyme activity.

Determination of subunit molecular weight by SDS: polyacryl-gel electrophoresis - Electrophoresis in polyacrylamide gel in presence of the anionic detergents has been used for the estimation of molecular weight of the proteins and their sub-units. The binding of dodecyl sulphate ions to proteins has been shown for several protein molecules and was assumed to be the basis of the separation of denatured protein upon SDS electrophoresis on polyacrylamide gel. Hence the individual charge pattern was totally changed by the binding by SDS anion rendering all the molecules negatively charged. In the present study, the method described by Weber and Osborne (1969) was used to determine the sub-unit and molecular weight of the protein. The mobility of the protein was calculated by using the expression:
The experiment was conducted along with the standard and the details of the run were explained in the experimental section. A linear relationship was observed when the electrophoretic mobilities were plotted against the logarithm of the molecular weight polypeptide chain. The mobility of the protein is calculated using the above expression as described by Weber and Osborn (1969).

Since sodium dodecyl sulphate and β-mercaptoethanol extensively disrupts hydrogen and hydrophobic bonds and S-S bridges, the same procedure was used widely to determine the subunit structure of proteins in recent years.

**Ultra centrifugation** - The measurements of sedimentation velocity were made with Spinco model E Ultracentrifuge with Schlieren optics. The experiments were conducted at 20°. 10 mg of pure protein were used. Pictures of sedimentation velocity pattern were taken at
intervals of 8 minutes after a speed of 59,600 rpm. From the value of $x$ obtained as a function of time, the sedimentation coefficient was calculated with the equation,

$$S = \frac{dx/dt}{w^2}$$

where $w =$ angular velocity in radius per second and is given by the equation $w^2 = (2\pi \text{ rps})^2$ (rps = revolutions per second).

**N-terminal analysis** — N-terminal amino acid was determined according to the procedure of Sanger (1951). Enzyme protein was treated under mild alkaline conditions (pH 9 - 9.5) with FDNB (1-fluoro-2,4-dinitrobenzene) for 4 hours. The unreacted FDNB was removed by ether. The DNP-protein was hydrolysed by heating at 110° for 8 hours in 7.5 M HCl in a sealed tube. The N-terminal DNP-derivative extracted with ether, solvent evaporated and the derivative dissolved in alcohol and used for chromatography.

**C-terminal analysis** — C-terminal amino acid was identified by using hydrated hydrazine according to the method described by Akabori (1952). The unreacted hydrazine was removed and the C-terminal amino acid was converted into dinitrophenol derivative by using FDNB
as described under N-terminal analysis using different solvent systems. The detailed procedure is given in experimental portion.

**Chromatography** - Paper partition chromatography was carried out using the ascending technique. Both Whatman No. 1 and 3 were used. Separation of sugars, organic acids and amino acids have been done using different solvents and spray reagents at room temperature (22° - 25° C) for 18 hours. The following solvent mixtures were used:

- **Solvent for sugars**:
  - n-butanol: acetic acid: water (4:1:1)

- **Solvent for organic acids**:
  - n-butanol: acetic acid: water (4:1:1)
  - phenol: water (4:1)

- **Solvent for BNP N-terminal amino acids**:
  - (i) 6 hours run for tertiary amyl alcohol on paper saturated with phthalate buffer, pH 6.6;
  - (ii) phosphate buffer, 0.5 M;

Different spraying agents were used to locate on paper organic acids, sugars and amino acids.
Spraying agents for organic acids:
0.05 per cent Bromophenol Blue in 95 per cent alcohol.

Spraying agents for sugars:
10 ml of 5 per cent Benzidine and 10 ml of 40 per cent trichloroacetic acid in acetic acid was made to 100 ml in alcohol and used for spraying.

Spraying agent for amino acids:
0.1 per cent Ninhydrin in acetone.

Photooxidation - Both Methylene Blue (MB) and Rose Bengal (RB) have been used with 100 watt tungsten lamp. Source of light was at a distance of 6 - 8 cm from the enzyme solution at a pH 9.6. Arrangements for continuous flow of water was made to maintain the temperature at 28° - 29°C. Photooxidation of enzymes has resulted in certain cases in a loss of activity which has been attributed to the destruction of tryptophan or histidine residues in the enzyme.

Aeration efficiency and dissolved oxygen - To determine dissolved oxygen, sulphite oxidation method recommended by Fernstran et al. (1944) was followed. Freshly prepared solution of sodium
sulphite (0.1 M) and copper sulphate (0.02 M) were placed in Erlenmeyer flasks and incubated on a rotary shaker. Flasks were removed at different intervals and dissolved oxygen was determined by titration of the residual sulphite. Aeration efficiency is expressed as millimoles of oxygen uptake per liter per minute.

pH was measured using a Universal pH meter. Cold centrifugation was carried out using the International Refrigerated Centrifuge Model HR-1. Collection of fractions during enzyme purification was carried out using an Emsmeeve fraction collector.