Chapter 2

Materials and Methods
Organism and culture conditions

Aspergillus terreus Thom was isolated from decaying straw on the basis of the clearance produced on cellulose-agar plates, and identified at the C.A.B. International Mycological Institute, Kew, Surrey, U.K (IMI Number 329789).

A. terreus was maintained on medium containing salts as described by Mandels and Weber (1969). This contained the following (per litre) - FeSO$_4$.7H$_2$O, 5.0mg; MnSO$_4$.H$_2$O, 1.6mg; ZnSO$_4$.7H$_2$O, 1.4mg; CoCl$_2$, 2.0mg; KH$_2$PO$_4$, 2.0g; (NH$_4$)$_2$SO$_4$, 1.4g; Urea, 3.0g; MgSO$_4$.7H$_2$O, 3.0g and CaCl$_2$, 3.0g. In addition to these, proteose peptone and non-crystalline cellulose (Sigmacell Type 100, Sigma Chemical Company, Missouri, USA) were added at concentrations of 0.05g% and 1.0g% respectively. Slants containing this medium were inoculated with spores of A. terreus and incubated at 28±2°C. Fully grown slants gave brown spores characteristic of this organism. Slants were sub-cultured every two weeks. Two-week-old slants were used for all experiments. A spore inoculum of approximately 10$^8$ spores per ml was prepared by washing out spores from the slants (in sugar tubes) using sterile Triton X-100 (0.01% v/v).

Cultivation under submerged conditions was done by inoculating spores into 100 ml of medium (in 250 ml Erlenmeyer flasks) containing the basal salts, proteose peptone, and a carbon source as required by the experiment (see below and Results & Discussions). Inoculated flasks were kept on a rotary shaker at 180 rpm at 28±2°C.
Preparation of mycelial pellets for immobilization

Mycelial pellets were prepared by inoculating spores into the above medium containing 0.2g% glucose as the sole carbon source. Flasks were harvested at the end of 72 hours. An inoculum size of $10^{11}$ spores per ml gave pellets of approximately 1 mm diameter.

Glutaraldehyde treatment of mycelia

72-hour-old mycelia were washed with sodium citrate buffer (pH 4.8, 0.01 M) (buffer A). These mycelia were then suspended in 2g% glutaraldehyde (obtained as 25% aqueous solution, Ferak Laborat, GMBH, West Berlin) in buffer A and kept on a rotary shaker (180 rpm) for 1.5 hours at 28±2°C. The mycelia were then washed extensively using buffer A to remove excess unreacted glutaraldehyde. These glutaraldehyde-treated mycelia were stored at 10°C and used within a week's time.

Treatments with other organic solvents were also done similarly; in case of nonmiscible solvents, the volume was made up with buffer A to give the required final concentration of solvent.

Assays for $\beta$-glucosidase activity

Assay using p-Nitrophenyl-$\beta$,D-Glucoside (PNPG) : This was done by the method of Berghem and Petterson (1973). The PNPG assay was used for the experiments in Chapter 3 (Induction and Secretion of $\beta$-Glucosidase in Aspergillus terreus; other assays were carried out using cellobiose (described below).

1.0 ml of enzyme, appropriately diluted in 0.05M citrate buffer, (pH 4.8) (buffer B) was added to 1.0 ml of 1.0 mM PNPG in
buffer B. The reaction mixture was incubated at 50°C for 10 minutes. Stopping of the reaction and formation of colour was done by addition of 2 ml of 1M sodium carbonate to the above reaction mixture at the end of 10 minutes of incubation. Absorbance was read at 400 nm.

One unit of β-glucosidase activity is defined as the amount of enzyme required to release 1 micromole of p-nitrophenol under the above assay conditions.

Assay using cellobiose: β-Glucosidase assay using cellobiose was done by the method of Sternberg et al. (1977). 1.0 ml of enzyme diluted in buffer B was added to 1.0 ml of 15 mM cellobiose in citrate buffer pH 4.8, 0.05M and the mixture was incubated at 50°C for 30 minutes. The reaction was terminated by incubating the tubes in boiling water for 5 minutes. The tubes were then cooled and the samples were frozen till glucose estimation was carried out.

Glucose liberated was estimated by the method of Dahlquist (1961) using the glucose oxidase peroxidase method. 100 ml of the reagent contained Na₂HPO₄ (1.38g), NaH₂PO₄ (0.726g), Glucose oxidase (Catalogue No. C 6125, Sigma Chemical Company, Missouri, USA) (32.0mg), Peroxidase (Catalogue No. P 8125, Sigma Chemical Company, Missouri, USA) (3.8mg) and 1.0 ml of orthodianisidine stock (20 mg orthodianisidine in 2 ml of methanol). For the estimation, 0.2 ml of appropriately diluted sample was added to 5.0 ml of the reagent, and incubated at 37°C for 20 minutes. 0.2 ml of 72% H₂SO₄ was added to all tubes in order to prevent further increase in colour formation. Colour formed was estimated at 435 nm.
1 unit of β-glucosidase activity is defined as the amount of enzyme required to liberate one nanomole of glucose per minute under the above assay conditions.

Assay for cell-associated β-glucosidase activity
Mycelia were filtered out on a cheese cloth and the buffer was allowed to drain out. 0.3g of these mycelial pellets were added to 25 ml of 150 mM cellobiose in citrate buffer (pH 4.8, 0.05M) in 100 ml Erlenmeyer flasks. The system was then incubated at 50°C for 30 minutes (unless otherwise mentioned in Results and Discussion). At the end of 30 minutes, the supernatant was separated from the assay mixture and the glucose liberated was estimated as described earlier (see above, cellobiose assay).

Study of the reusability of cell-associated β-glucosidase activity
The system for operational stability was the same as for the mycelial β-glucosidase, but each cycle was of 24-hour duration. The flasks were incubated at 50°C. At the end of each cycle, the supernatant was taken for glucose estimation and the mycelium was washed with buffer and resuspended in fresh substrate.

Study of the effect of pH
The buffers used were as follows— for the pH range of 3.0 to 7.0, citrate-phosphate buffer was used; for pH values 8.0 and 9.0, Tris-HCl buffer was used. All buffers were used at 0.05M concentrations. For studying the effect of pH on the activity, the assays were directly carried out in the appropriate buffer. In order to study the effect of pH on stability, the mycelia were incubated in the corresponding buffer at 50°C for thirty minutes.
in absence of substrate; the mycelia were then cooled, washed with 0.05M citrate buffer (pH 4.8) and then assayed for activity as described earlier.

**Continuous cellobiose hydrolysis in a column reactor**

A jacketed glass column (with a height of 27 cm and an internal diameter of 3.5 cm) was maintained at 50°C using a water-circulator (Multitemp, LKB, Sweden). 50g of mycelium was weighed (as described above in Assay for mycelial β-glucosidase) into the substrate (15 mM cellobiose in buffer B), and the flow of substrate was started from a reservoir (Figs. 2.1a and 2.1b). The substrate from the reservoir was supplied in such a way that fresh substrate passed upward through the mycelial bulk. Reacted effluent was collected through the top of the column. As the system was air-tight, the flow could be regulated using a pinch cork at the outlet tubing. A flow-rate of 0.5 ml per minute was maintained and fractions collected at various intervals were used for estimating the glucose released.

**Cellobiose hydrolysis in batch mode**

Cellobiose hydrolysis in unstirred batch mode was done by taking 10ml of the required concentration of cellobiose solution (in citrate buffer, 0.05M, pH 4.8) in sugar tubes. Glutaraldehyde treated mycelia were added to the solution till all the liquid was in contact with the mycelia. The tubes were incubated at 50°C and samples were withdrawn at various intervals as described in Results and Discussion.
Fig. 2.1a Column reactor used for celllobiose hydrolysis.

Fig. 2.1b Schematic diagram of column reactor used for celllobiose hydrolysis.

a = inlet for substrate
b = outlet for product
c = outlet for circulating water
d = inlet for water
Paper chromatography of cellobiose hydrolysis products

Chromatography of the sugars was done using a system consisting of n-Butanol : Pyridine : Water in the ratio of 6:4:3. At the end of the run, the paper was air-dried in the fume-hood itself and then sprayed twice with silver nitrate in acetone (200 ml of acetone contained 1 ml of 20% AgNO₃). After drying again, the paper was sprayed with 1.4g NaOH in 100 ml methanol. (All reagents were prepared fresh). Sugars were visualised as yellowish brown spots.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Davis (1964). Electrophoresis was carried out at a constant current of 20mA in 7.5% gels of 14 x 16cm size (LKB 2001 vertical electrophoresis system, LKB, Sweden). During the run gels were maintained at 15°C using a water circulator.

SDS-PAGE was carried out by the method of Laemmli (1970). Enzyme samples for activity staining were prepared by incubating the enzyme in sample buffer for 2 minutes at 100°C or 2 hours at 50°C as described in the text.

Staining for β-glucosidase activity

Post-electrophoretic gels were transferred to 0.1 M citrate buffer containing 2mg% 4-methylumbelliferyl-β-D-glucoside (MUG) (Sigma Chemical Company, Missouri, USA). This method is a modification of the method used earlier by Sprey and Lambert (1983). These workers carried out activity staining for β-glucosidase by spraying the surface of post electrophoretic
gels with 10mM solution of MUG in 0.05M acetate buffer.

Incubation in the solution for 3 to 5 minutes at 30°C led to the formation of 4-methylumbelliferone which is visualized as a blue fluorescence under ultra violet light. (In case of SDS-PAGE, gels were washed overnight with buffer A before staining).

**Extraction of the enzyme from the mycelia**

Crude enzyme for enzyme purification was obtained from 72-hour old mycelia grown on 0.2g% glucose. Mycelia were disrupted by sonication at 4°C using a sonicator (Vibronics Ultrasonic Processor P2, Vibronics Pvt. Ltd., Bombay, India). Sonication was carried out in bursts of 30 seconds till no intact mycelia could be detected microscopically. Extraction was carried out into 0.01M Tris HCl buffer containing 1mM EDTA and 1mM sodium azide. The cell-debris was removed by centrifugation (10,000g for 10 minutes at 10°C).

**Purification of β-glucosidase**

The extracted crude enzyme was then dialysed against the same extraction buffer with three changes over a period of 24 hours. The dialysed crude enzyme was concentrated 10-fold by reverse dialysis against polyethylene glycol-20,000 (Sigma Chemical Company, Missouri, USA) and loaded on 3 mm thick, non-denaturing polyacrylamide gel and the proteins were separated by electrophoresis using a constant current of 40mA. The temperature was kept constant at 15°C. At the end of the run, (when the dye-front emerged from the gel) the gel was stained for β-glucosidase activity as described earlier. The blue band of fluorescence (seen under ultra violet light) was cut out from the
rest of the gel. The enzyme was eluted from the gel by either simply allowing the enzyme to diffuse out into buffer or by electroelution as described below.

Electroelution of β-glucosidase from polyacrylamide gels

The glass plates for preparatory PAGE were refixed in position (using 3 mm spacers as before). The lower part of the plates were sealed with 1 g% agar in Tris-HCl buffer (pH 8.9, 7.5 ml of stock in 60 ml distilled water) (Fig. 2.2). The space above this was filled with 35 ml of the Tris-HCl buffer of the above composition. The gel strip was inserted over this buffer without any air bubbles below the gel. The gel was fixed in position by layering a 1 mm layer of agar solution over the gel. The upper space was filled with electrode buffer and electrophoresis was carried out for 2 hours, at a constant current of 30 mA at a temperature of 15°C. At the end of 2 hours, the upper layer of buffer was removed and the buffer below the gel was collected and dialysed against buffer A to remove all traces of Tris-HCl buffer. This purified enzyme was used for further studies.

Glutaraldehyde treatment of purified β-glucosidase

2.0 ml of purified enzyme (eluted from PAG) was mixed with 2.55 ml distilled water, 0.25 ml of 0.25 ml of 1M citrate buffer (pH 4.8) and 0.2 ml of 25% glutaraldehyde. This mixture was incubated at 28°C for 1 hour. Excess, unreacted glutaraldehyde was removed by extensively dialysing against 0.05M citrate buffer, at 4°C, with frequent changes of the buffer.
Fig. 2.2 Set-up for electroelution of β-glucosidase:

- a = glass plates;  
- b = 3 mm spacers;  
- c = electrode buffer;  
- d = gel strip;  
- e = eluent buffer;  
- f = agar seal.
Protein Estimation

Protein estimations were done by the method of Lowry et al. (1951).

Staining for proteins on PAGE

Silver staining was carried out by the method of Gooderham (1984). Post electrophoretic gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 2 to 16 hours and then transferred to 20% (v/v) methanol, 7% (v/v) acetic acid for another 2 to 16 hours. The gels were then incubated in 10% glutaraldehyde solution for 30 minutes, and washed overnight with distilled water.

The gels were then incubated in ammoniacal silver nitrate for 15 minutes (ammoniacal silver nitrate was prepared as follows: to approximately 170 ml of distilled water, 2.8 ml of concentrated ammonia solution and 3.75 ml of 1N NaOH solution were added. A volume of 8 ml of 20g% silver nitrate solution was then slowly added with vigorous shaking. The solution was then made upto 200 ml with distilled water).

Proteins were visualised by transferring the gels to a solution of 0.005g% citric acid and 0.019% of 38% formaldehyde till the colour developed (black to brown bands). The increase in intensity was stopped by quickly adding 20% methanol, 7% acetic acid when the required contrast was attained.

Coomassie stain was prepared by dissolving 250 mg of Coomassie Blue R in 50 ml methanol in a clean dry flask. 10 ml acetic acid was then added and the volume made to 100 ml with distilled water. Gels were left in the stain for 2-16 hours and then
destained using a solution of 10% methanol and 7.5% acetic acid.

**Glycoprotein staining**

Glycoprotein staining was done by the Periodic acid - Schiff method (Zacharius et al., 1969). Gels for glycoprotein staining were fixed in 12.5% TCA for 1 hour, rinsed and then transferred to 1% Periodic acid solution for 1 hour. Gels were then washed overnight with distilled water to remove all traces of excess periodic acid. Subsequent incubation in Schiff's reagent (Sisco Research Laboratories, Bombay, India) gave pink to violet bands characteristic of glycoproteins.