CHAPTER II

MATERIALS AND METHODS
Cultures:

The plus (+) (NRRL 2895) and the minus (-) (NRRL 2896) strains of Blakeslea trispora were obtained from Dr. A. Ciegler, U.S. Department of Agriculture, Peoria, Illinois, U.S.A.

Culture Maintenance: The cultures were individually maintained on the synthetic medium-agar slopes as described by Anderson et al. (1). The medium contained (per litre) glucose, 20g; L-asparagine, 1.0g; KH\(_2\)PO\(_4\), 1g; MgSO\(_4\).7H\(_2\)O, 0.5g; thiamine HCl, 1 mg; traces of Fe\(^{++}\), Zn\(^{++}\) and Mn\(^{++}\) and Difco agar, 18 g. The pH of the medium was adjusted to 6.2 with NaOH and sterilized at 10 lbs/sq. in. for 20 minutes. Subculturing was done every fifteen days and the slants were kept at 28\(^0\) + 2\(^0\)C temperature.

Media for cultivation:

A. A synthetic mucor medium as described by Hesseltine and Anderson (2) was used primarily to evaluate the effect of various medium constituents on the \(\beta\)-carotene synthesis, \(\beta\)-glucosidase production and the growth of B. trispora. The medium consisted of (per litre) glucose, 40g; L-asparagine 2g; KH\(_2\)PO\(_4\), 0.5g; MgSO\(_4\).7H\(_2\)O, 0.25g; thiamine HCl, 10 mg; pH 6.2.

The influence of the nature and the concentration of the various carbon and nitrogen sources was studied by substituting glucose or asparagine of SMM by the respective carbon or nitrogen source as described in the text (Chapter III).
The complex nitrogen sources used viz. castor seed cake, cotton seed cake, ground nut cake and cattle feed were purchased from the local market. A 10g% slurry of either of these nitrogen sources was hydrolyzed (0.4N HCl) at 15 lbs/sq. in. for 90 min. The nitrogen sources were included in the medium on the gram wt. basis, for example; 20 ml of this hydrolyzate corresponds to 2g% of the nitrogen source. The cattle feed is a mixture of various deoiled seed cakes. The chemical composition of the cattle feed used in the present experiments was (as provided by the manufacturers Godrej Pvt. Ltd, Bombay): Crude protein, 24%; Crude fat, 4%; Crude fibre, 9%; Calcium, 1%; Phosphorus, 0.7% and acid soluble ash, 3.5%. The effect of inorganic phosphate concentration was studied by varying the concentration of KH₂PO₄ in the medium (Chapter - V). Unless otherwise mentioned, 100 ml of medium after distribution in 250 ml Erlenmeyer flasks (Corning) were sterilized at 10 lbs/sq. in. for 20 minutes.

B. Fermentation medium

The fermentation medium used to study the influence of various cultural conditions (Chapter - VI) and during the scale-up of the fermentation process consisted of (per litre) soluble starch, 10g; cattle feed hydrolysate, 20g; KH₂PO₄, 10g; MgSO₄·7H₂O, 0.25g; thiamine HCl, 10 mg.
The pH of the medium was adjusted to 6.2 with NaOH and the media sterilized at 15 lbs/sq. in. for 30 minutes.

Inoculum preparation and cultivation

The mycelial growth of either mating type from 5-6 day old agar slant was used to inoculate 100 ml of SMM. Both the strains (+ and -) were grown separately for 48 h at 28° ± 2°C on a gyrotary shaker (180 rev./min). For experiments in a fermentor (Chapter VI) fermentation medium was used instead of SMM for the inoculum preparation. Equal volumes of each mating type were combined, washed thoroughly with distilled water and transferred aseptically to a Sorvall omni mixer and macerated to make a homogenous suspension. A 10-ml aliquot of the homogenate was used to inoculate each 100 ml of the growth medium. As both the mating types were combined before the maceration, the resulting cultures were referred to as mated cultures. The mated cultures were used in all the experiments. The cultures were incubated for 5 days at 28° ± 2°C on a gyrotary shaker (180 rev./min) unless otherwise specified.

Growth in the fermentors: During the scale-up experiments either 1 litre LKB-Biotech fermentor or a 100-litre fermentor was used.

The LKB-Biotech model FE 007 (made in Sweden) with 1 litre capacity was used (Fig. 1). The
Fig. - 1: LKB-Biotech 1 lit. fermentor (Stockholm, Sweden).
fermentations were conducted at 500 ml operating volumes. A 10% inoculum was used in each experiment and hence the final volume in each fermentor was 550 ml. All the fermentations were carried out at the maximum aeration efficiency of the fermentor.

A detailed description of the 100-litre stainless-steel fermentor is given in Fig. 2. In all the experiments, 50-litre medium and 10% inoculum was used. Thus the final volume in each fermentor was 55 lit. All the experiments were carried out at 28° ± 1°C and with an impeller speed of 424 r.p.m. The air supply in the fermentor is varied according to the experiment as described in the text.

Silicone defoamer (Metroark Pvt. Ltd., Calcutta) was added after every 48 h of the fermentation (1 ml in Biotech fermentor while 10 ml in 100 litre fermentor).

Extraction and estimation of carotenoids.

The cultures were filtered, the mat was washed thoroughly with water and pressed dry. The mycelia were then ground in acetone: diethyl ether (1:1 v/v) mixture. The mixture was filtered and the residue was reextracted till it was colourless. The residue was then collected, dried at 50°C and weighed. The solvent extract was washed with cold distilled water and dried over anhydrous sodium sulphate. The total carotene content was estimated as β-carotene using $E_{1%}^{1cm}$ value of 2500 at 450 nm as described by Davies (3).
FIGURE - 2: Schematic diagram of a 100-litre (pilot) fermentor.

Fermentor details:

(a) Diameter of the fermentor --- --- --- 15"
(b) Depth of the unit --- --- --- --- --- 48.5"
(c) Length of the stirrer rod from the top of the unit --- --- --- --- 45.5"
(d) Distance between the bottom of the unit and the stirrer end (b-c) --- --- 3"
(e) Depth of the sparger tip from the top of the unit --- --- --- --- 47"
(f) Distance of the sparger tip from the bottom of the unit (b-e) --- --- 1½"
(g) Distance between the sparger tip and the stirrer end (e-c) --- --- 1½"
(h) Distance between two impellers --- --- 10½"
(i) 1st blade centre from the end of the stirrer rod --- --- --- 1"
(j) 1st and 2nd blade c/c distance --- --- 5.25"
Growth determination: After extraction of carotenoids, the lipid-free mycelia were dried at 50°C in an oven and weighed. In the experiments where carotenoids were not extracted, the mycelial growth was harvested by filtration and dried at 50°C till constant weight. The growth is expressed as grams mycelial dry weight.

Enzyme preparation:
(a) For the extracellular enzyme activity, the culture filtrate was obtained by filtration of the cultures through four layers of cheese cloth. The culture filtrate was dialyzed against distilled water at 4°C for 24 h and was used either directly as the enzyme preparation or was subjected to further purification.
(b) For intracellular enzyme assays, the mycelia were harvested at appropriate times by filtration at 2-5°C and frozen. The frozen cells were ground manually at 2-5°C along with powdered glass (1:1 w/w) in an unglazed, precooled mortar and pestle till almost complete cell breakage ensued and an extract (10% w/v) was prepared with 0.01 M Tris-HCl buffer (pH 7.2). The extract was then centrifuged in a refrigerated centrifuge (-10°C) at 15,000 x g for 20 min. The supernatant designated as the cell free extract was either directly used as the enzyme preparation or was dialyzed against distilled water at 4°C for 24 h and then used as the enzyme preparation.

Purification of β-glucosidase: All the operations were carried out at 0 to 4°C. (i) Ammonium sulphate
fractionation: To the culture filtrate \((\text{NH}_4)_2\text{SO}_4\) powder was slowly added with constant stirring to 70% saturation and after 6 h incubation in the cold the precipitate was removed by centrifugation \((20,000 \times g, 20\) min) and discarded. The \((\text{NH}_4)_2\text{SO}_4\) concentration of the supernatant was adjusted to 100% saturation and left overnight. The precipitate, which contained most of the \(\beta\)-glucosidase, was collected by centrifugation \((20,000 \times g, 20\) min) and dissolved in a minimum amount of 0.05 M sodium phosphate buffer \((\text{pH} 7.0)\) and residual \((\text{NH}_4)_2\text{SO}_4\) was removed by dialysis against 2 l of the same buffer for 24 h. (ii) Gel filtration: An aliquot of the \((\text{NH}_4)_2\text{SO}_4\) fractionation was applied onto the top of the Sephadex G-200 column \((34.0 \times 2.2\) cm) previously equilibrated with 0.05 M sodium phosphate buffer \((\text{pH} 7.0)\). 35 fractions, each containing 5 ml were collected and assayed for \(\beta\)-glucosidase activity.

The active pooled fractions of \(\beta\)-glucosidase obtained from the Sephadex G-200 column (marked A in Fig. 1, Chapter - IV) was applied to a DEAE-Sephadex A50 column \((12.0 \times 1.5\) cm) previously equilibrated with 0.05 M sodium phosphate buffer \((\text{pH} 7.0)\). The column was washed with 150 ml of the buffer and subsequently eluted with a linear NaCl gradient increasing from 0 to 0.5 M in 250 ml of the same buffer. The active fractions (marked B in Fig. 2, Chapter - IV) were pooled and dialyzed against 0.05 M sodium phosphate buffer and used as purified preparation for further studies.
Protein determination: Protein was estimated by the method of Lowry et al (4) using bovine serum albumin (Sigma) as the standard protein. In monitoring column chromatography effluents, protein was determined by recording the absorbance at 280 nm on Beckman DU 2 spectrophotometer.

Molecular weight: Molecular weight estimation of the purified enzyme preparation was carried out by gel filtration on Sephadex G-200 column as described by Andrews (5), using standard proteins of known molecular weights, viz. catalase (MW 232,000), \( \beta \)-globulin (MW 160,000), lipoxidase (MW 108,000), Haemoglobin (MW 65,000) and Trypsin (MW 24,000).

Enzyme Assay: (a) \( \beta \)-glucosidase: The method described by Wood (6) was followed for the estimation of \( \beta \)-glucosidase activity. In a typical assay, \( \beta \)-nitrophenyl-\( \beta \)-D-glucoside (PNPG), 1 mM in 50 mM sodium acetate buffer (pH 5.0) and appropriately diluted enzyme were incubated together at 50°C for 1 h. The reaction was terminated by adding 4.0 ml of 0.4 M glycine-NaOH buffer (pH 10.6) and the amount of \( \beta \)-nitrophenol liberated was measured spectrophotometrically at 430 nm.

One unit of \( \beta \)-glucosidase activity is defined as the amount of enzyme required to liberate 1 \( \mu \)mole of \( \beta \)-nitrophenol per min at 50°C.

(b) Cellobiase activity: A method described by Wood (7) was
followed to determine the cellobiase activity. The incubation mixture contained acetate buffer pH 5.0, 100 μmoles, different concentrations of cellobiose (as described in the text) and an appropriately diluted enzyme and distilled water to give a total volume of 3.0 ml. After incubation at 50°C for 1 h, the tubes were immersed in the boiling water bath for 5 min and the glucose released from cellobiose was estimated by adding 3.0 ml of a glucose oxidase-peroxidase reagent (prepared by adding: (a) 12 mg of glucose oxidase, (b) 0.5 mg of peroxidase and (c) 0.5 ml of a 1% (w/v) solution of o-dianisidine in 95% ethanol, to tris buffer, pH 7.0 to give final volume of 100 ml.) to 1.0 ml sample and the solution incubated for 1 h at 37°C in dark. The enzyme reaction was terminated by the addition of 4.0 ml of 25% H₂SO₄ and the glucose was calculated from the absorbancy at 515 nm.

One unit of cellobiase activity is defined as the amount of enzyme that releases 1 μmole of glucose per min at 50°C.

(C) Phosphatase activity:

A method described by Torriani (9) was followed to determine the acid and the alkaline phosphatase activities in a typical enzyme assay, 2.5 mM p-nitrophenyl phosphate, 1mM magnesium acetate, an appropriately diluted enzyme and 200 μmoles of buffer in total system of 4 ml were incubated
at 30°C for 1 h. For the acid phosphatase acetate buffer, pH 5.0, and for alkaline phosphatase activity tris-HCl buffer, pH 8.5 was used. The reaction was terminated by adding 2 ml of 1 M Na₂CO₃ and the amount of p-nitrophenol released was estimated spectrophotometrically at 410 nm.

One unit of the phosphatase activity is defined as the amount of enzyme required to release 1 μmole of p-nitrophenol per min at 30°C.

Specific activity of an enzyme is defined as units per mg protein.

Effect of pH on the enzyme activity and stability:
Effect of pH on p-glucosidase activity was studied by using different buffer system - Sodium acetate (pH 3.0 - pH 5.5), Sodium phosphate (pH 5.0 - pH 8.0), Tris-HCl (pH 7.0 - pH 8.5) and Barbital (HCl) (pH 8.0 - pH 9.5) in the assay systems. The stability of the enzyme was tested by preincubating the enzyme solution adjusted to various pH levels with the help of equal amounts of appropriate 0.1 M buffer, for various time intervals at 30°C. The residual activity in the appropriately diluted sample was measured under standard assay conditions.

Buffers:
All buffers and other solutions were prepared using glass distilled water, as described by Gromori (9).
Gel electrophoresis

(a) Polyacrylamide gel electrophoresis of p-glucosidase and the phosphatases was performed according to the procedure of Davies (10). Aliquots containing 20-200 μg protein were subjected to electrophoresis in 7.5% polyacrylamide gel. Electrophoresis was conducted for two hours, in tris-glycine buffer, pH 8.3, using a constant current of 3 mA per tube. Bromophenol blue was used as the reference point of migration. The gels were stained with 0.1% Coomassie blue for two hours and destained by repeated washing with a methanol, acetic acid mixture (methanol 5%, acetic acid, 7%).

(b) Sodium dodecyl sulfate (SDS) - gel electrophoresis containing 7.5% acrylamide and 0.1% SDS was carried out as described by Weber and Osborn (11).

(c) The electrophoretic fractionation of the isozymes of the acid and the alkaline phosphatase was performed on 7.5% polyacrylamide gels. The cell free extract was applied (< 0.2 ml) to the top of the gel just before electrophoresis. The current applied was 5 mA per gel and the electrophoresis was completed at 5°C in 75 min. For detection of the acid phosphatase (acetate buffer, pH 5.0) and the alkaline phosphatase (Tris-HCl buffer pH 8.5) activities on the gel, the method described by Mattoo and Shah (12) was followed. The gels were incubated at 30°C with a reaction mixture containing 0.2M buffer, 0.6 mM
lead acetate, 1 mM magnesium acetate and 10 mM sodium 
β-glycerophosphate. At the end of the incubation (4-6 h) 
the gels were first washed in distilled water and then in 
7% acetic acid for 2 min, and finally immersed in a 1% 
solution of sodium sulphide for 5 min. The bands of 
enzyme activity turned black against faint yellow to 
white background.

Saccharification of Avicel by mixed culture filtrates:
The culture filtrate of a wild type Trichoderma viride 6a 
cultured as described by Mandels' and Reese (13) with 2g% 
cellulose powder (Centron) in the medium as a carbon source 
was obtained by low speed centrifugation. A total of 2 ml 
of a 6% suspension of Avicel in 0.2M sodium acetate buffer 
(pH 5.0) was added to 2 ml of a mixture of B. trispora 
culture filtrate and T. viride culture filtrate. The 
suspension was incubated at 50°C. After incubation, the 
amount of reducing sugar was determined by the DNSA method 
as used by Mandels et al (14). Sodium azide (10 μg/ml) 
was added to all the enzyme solutions to prevent microbial 
growth.

Chemicals. All the fine chemicals including D-cellobiose, 
β-nitrophenyl-β-D-glucoside, various substrates for the 
β-glucosidase assay, standard proteins for molecular weight 
determination etc., were obtained from Sigma Chemicals Co. 
(Missouri, U.S.A.). Other chemicals used during the work 
were of either analytical grade or of reagent grade.
Analytical Methods.

Estimation of total carbohydrates: Total carbohydrates of the fermentation medium were determined by phenol-sulfuric acid method of Dubois et al (15) in terms of glucose. The Glucose was estimated by the glucose oxidase method of Dahlquist (16).

Estimation of Total nitrogen:

Total nitrogen was measured by micro-Kjeldahl method (17).

Phosphate estimation: Estimation of inorganic phosphate was done by the method of Fiske and Subba Row (18).

Aeration rate: The rates of aeration were measured by the sulfite oxidation method of Cooper et al (19).
REFERENCES CITED: