CHAPTER II

MATERIAL AND GENERAL METHODS
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Organism:

*Alternaria brassicae* (Berk.) Sacc. isolated from severe leaf spot of mustard (*Brassica campestris* L.) was selected to investigate whether cellulolytic enzymes are produced by the organism and whether they are involved in disease development.

Medium:

Czapek-Dox agar supplemented with alfalfa seed decoction was employed for maintenance of the fungus.

Composition of the medium:

Sucrose 1.5 %, NaNO₃ 0.2 %, KH₂PO₄ 0.1 %, MgSO₄·7H₂O 0.5 %, KCl 0.05 %, FeSO₄ 0.001 %, Agar 2.0 % and alfalfa seeds 100 g/litre were taken in a 1500 ml conical flask. It was sterilized under 15 pounds pressure for 20 minutes. The medium was acidified by adding 2.5 ml of sterilized NHCl per 100 ml to obtain pH 4.5 of the medium in order to check the growth of bacteria.

Sub-culturing was done once a month for maintaining the organism on Czapek-Dox.

In preliminary experiments glucose nitrate (GN) was used as a basal medium for control in cellulase production study.
Composition of the medium:

Glucose 1%, KN0₃ 0.25%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%.
The composition of other liquid media used in cellulase production have been given later.

Culture vessels:

All glassware were thoroughly cleaned with acid dichromate cleaning mixture, hot tap water, rinsed with distilled water and dried completely before use. Liquid media (25 ml) were placed in each 250 ml Erlenmayer conical flask and autoclaved in the usual way.

Method of inoculation:

Spore suspension was prepared by adding 10 ml sterile distilled water to ten days' old Czapek-Dox supplemented with alfalfa seed decoction agar slope culture and 5 drops of it were used as inoculum in all experiments. Spore suspension was standardised with the help of a Haemocytometer to contain 5x10⁵ spores per microscopic field. All treatments were in triplicate and results have been presented after repeating each experiment reported here.

Incubation:

Cultures were incubated at 25±1°C and this variation in temperature was recorded during the years under study. In all experiments the cultures were incubated for five days except stated otherwise.
Method of assessing growth and pH:

The mat of mycelium was removed onto a previously weighed Whatman No.1 filter paper kept overnight at 60°C and the dry weight of the mycelium was then determined.

The pH values of media and filtrates were determined with a pH meter.

ENZYME STUDIES

*Alternaria brassicae* was taken to study its capacity to produce cellulolytic enzymes under identical conditions. It was employed to understand the effect of various physical and nutritional conditions on the in-vitro, in-vivo and intracellular production of cellulolytic enzymes. Properties of the different components of the enzyme were also studied.

**Media:**

The basal salt medium was prepared with the following composition (N-medium):

\[ \text{KNO}_3 \ 0.25\%, \ \text{KH}_2\text{PO}_4 \ 0.1\%, \ \text{MgSO}_4\cdot\text{7H}_2\text{O} \ 0.05\% \]

1% of various cellulosic carbon sources like Na-carboxymethyl cellulose (CMC), cellulose powder (C), cotton (Co), filter paper (F), cellobiose (Cb), and glucose (G) as a non-cellulosic carbon source were added separately to prepare different media.
The enzyme activities of the different culture filtrates differed considerably, but the following medium gave active preparation:

Carboxymethyl cellulose (CMC) or cotton, filter paper or cellulose powder or cellobiose 1%, L-rhamnose 0.5%, \( (NH_4)_2HPO_4 \) 0.5%, KH\(_2\)PO\(_4\) 0.1%, MgSO\(_4\)·7H\(_2\)O 0.05% and BaCl\(_2\) 0.02%.

Twenty-five ml of the medium were poured in each 250 ml conical flask, sterilized, inoculated as usual and incubated at 25±1°C. The fungus required 5 days, 10 days and 15 days incubation period on CMC, cotton and filter paper, and cellulose powder and cellobiose medium respectively to produce active enzymes. The culture filtrates from a number of cultures were then mixed and centrifuged at 5000 rpm for 10 minutes to obtain cell-free solutions. They were stored in 25 ml quantities under a few drops of toluene in screw cap glass bottles at 5°C and used as required. The dry weight of the mycelium and pH of the filtrates were also determined in the way already given earlier.

In some experiments this crude enzyme preparation was partially purified by precipitation or by dialysis. Details of the methods which were used will be given later.

**Determination of loss of coherency activity:**

In this method discs of 0.3 mm in thickness and 6.0 mm in diameter were cut with a punching machine from Whatman
filter paper No.1. Five such discs were placed in 3 ml of enzyme solution (culture filtrate) + 2 ml of 0.2M citrate-phosphate buffer of respective pH and subjected to slight tension by hand at every five minutes until the coherence was lost. The mean time for loss of coherence was noted and taken as the reaction time (R.T.) in minutes. Macerating activity (M.A.) is expressed as $M.A. = \frac{1000}{R.T.}$. Tests were carried out at the laboratory temperature of 25 to 30°C and unless stated otherwise at pH 5.6 which was the optimum pH for activity. In the case of cotton fibres, each fibre measured 0.4 mm thick and 5.0 cm in length, five such fibres were placed in 3 ml of the culture filtrate along with 2 ml of 0.2M citrate-phosphate buffer of pH 5.6. These were subjected to slight tension by hand after $\frac{1}{2}$, 1, 2, 4, 8 and 12 hr intervals until coherence was lost. R.T. and M.A. were calculated as above and expressed in hours.

Viscometric method:

When derivatives of cellulose like CMC are attacked by cellulolytic enzymes, they are degraded to compounds of low molecular weight and therefore their viscosity is reduced. These enzymes hydrolyse $\beta$-1,4 glucosidic linkages and split the aggregates of reducing group molecules which are responsible for the high viscosity of these substances. The detection and assessment of cellulolytic activity is usually based on viscosity measurements. Experiments on the
viscosity reducing activity of the culture filtrate of *Alternaria brassicae* with CMC were conducted and results are reported in the respective sections.

Carboxymethyl cellulose (CMC) with the following chemical nature was used as substrate:

- The average molecular weight of CMC (viscometric method) = 1,09,700
- The degree of substitution (DS) = 0.595
- The average degree of polymerization (DP) = 523.00

The substrate-enzyme mixture had the following composition:

- 0.5% CMC 5 ml
- Active crude culture filtrate 3 ml
- 0.2M citrate-phosphate buffer 2 ml

In all experiments active crude enzyme was used to determine its viscosity reducing properties. Viscosity measurements were carried out with an Ostwald Viscometer.

The tube containing the substrate solution and an Ostwald Viscometer were thoroughly cleaned and dried beforehand. Three ml of enzyme solution and 2 ml of buffer were then added to the substrate and simultaneously a stop watch started. The substrate-enzyme mixture was quickly stirred. Viscosity readings were taken at 0, 5, 10, 20 and 30 minutes after adding the enzyme solution.

Viscosity, represented as the time of flow in seconds, of the reaction mixture through the capillary of the
viscometer, was plotted against time in minutes. In all cases smooth curves were obtained indicating that loss of viscosity was directly proportional to the time for a given volume of mixture to flow through the capillary of the viscometer.

From each viscosity/time curve the percentage of loss of viscosity was calculated in the following way:

\[ V_0 = \text{Viscosity of substrate-enzyme mixture at 0 hours.} \]
\[ V_t = \text{Viscosity of substrate-enzyme mixture at } 't' \text{ minutes after adding the enzyme.} \]
\[ V_w = \text{Viscosity of water.} \]

Total possible loss of viscosity \[ = V_0 - V_w \]

Viscosity loss due to the action of enzyme \[ = V_0 - V_t \]

\[ \% \text{ viscosity loss due to the action of enzyme} = \frac{V_0 - V_t}{V_0 - V_w} \times 100 \]

This substrate-enzyme mixture was used to make viscosity/time measurements and the per cent viscosity loss of CMC was calculated with the formula given above from viscosity measurements up to 30 minutes. The maximum viscosity loss at a given time was considered as due to 100 units of enzyme. Accordingly, units of enzyme activity were calculated at a given time in various treatments and graphs were plotted.
Estimation of reducing groups by Somogyi's method:

Preparation of copper reagent:

(a) One litre solution contains:

i. 28 g of anhydrous Na₂HPO₄ (70.6 g of Na₂HPO₄·12H₂O)

ii. 100 ml of 1N NaOH

iii. 40 g of Rochelle salt

iv. 8 g of CuSO₄·5H₂O and

v. 180 g of anhydrous Na₂SO₄.

Sodium phosphate (Na₂HPO₄) and tartarate (Rochelle salt) were dissolved in about 700 ml of distilled water, NaOH solution added and then 80 ml of 10 % CuSO₄·5H₂O added with stirring, finally Na₂SO₄ was added and made to one litre.

(b) 1N KI₃ solution (potassium iodate) was prepared

(c) 2.5 % KI (potassium iodide) was prepared as required

(d) 0.005N sodium thiosulphate (hydrous) and

(e) 2N H₂SO₄ (approximately) were prepared in separate containers.

Procedure for assay:

5 ml of hydrolysate in case of swollen cellulose and 1 ml in case of carboxymethyl cellulose (CMC) or cellulbiose were mixed with 5 ml of the copper reagent treated with 1N KI₃ (200 ml Cu⁺⁺ reagent + 5 ml of KI₃) in a 2.5x20 cm Pyrex tube and heated for 10 minutes in boiling water and then cooled. An approximate amount (2.5 ml) per test tube
of 2.5% KI solution was added and the mixture was acidified with 2N H₂SO₄ (1.5 ml per test tube). The liberated I₂ was titrated with 0.005N sodium thiosulphate.

1 ml of 0.005N Na₂S₂O₃ = 0.135 mg of glucose.

**Assay of cellulase activity:**

Cellulase activity was estimated on the basis of reducing sugar formed by the action of enzyme on carboxymethyl cellulose unless otherwise stated. The assay solution consisted of 1 ml of 1% CMC, 2 ml of 0.1M citrate-phosphate buffer of different pH values like 2.6, 3.6, 4.6, 5.6, 6.6, 7.6 and 8.6 (saturated with antiseptic toluene) and 1 ml culture filtrate. The reaction mixture was placed in 25 ml screw bottle, a drop of toluene added to it and incubated at laboratory temperature 25-30°C for 1/2, 1, 2 and 4 hours in the stationery state. After each incubation period 1 ml of the hydrolysate was assayed for reducing sugar according to the micromethod of Somogyi (1945). After necessary corrections for controls cellulase activity was expressed in terms of millilitres of 0.005N Na₂S₂O₃ solution required in the titration of reducing sugar per 1 ml of hydrolysate.

**In-vivo production of cellulase:**

Detection of cellulase in the rotted potato tuber was carried out. Fresh and healthy potato tubers were brought from the market. These were surface sterilized with
rectified spirit in the inoculation room already sterilized with ultra-violet light. After sterilization, 2" deep cavities were made into each tuber with the help of a sterile cork-borer No.4 having a diameter of 8.0 mm. Five drops of spore suspension were placed in each cavity and the potato cylinders were replaced. Potato tubers were inoculated similarly with sterile distilled water and used as control. Tubers were incubated for 10 days at 25±1°C. All tubers were placed in the sterilized tin box to avoid contamination. After incubation period, the tubers were cut transversely to scrap the rotted portion and its weight was recorded. It was later crushed in a mortar with pestle by adding distilled water in 500:1 (mg/ml). The extract was filtered with fine cloth and then centrifuged at 5,000 rpm for 10 minutes to make cell-free. The pH of the supernatant fluid was noted by a pH meter and it was then used as enzyme solution and enzyme activity was determined by the usual assay methods.

STATISTICAL METHODS

Statistical analysis of growth was carried out in order to note down the significance of various treatments. Statistical methods for determination of standard deviation, standard error and critical difference between treatments were followed after Panse and Sukhatme (1961) and Anderson and Zelditch (1968).
Values for standard deviation between growth obtained in various treatments were calculated according to the following formula:

\[ S^2 = \frac{\sum X^2 - (\sum X)^2}{N - 1} \]

\[ S = \sqrt{S^2} \]

The standard error of mean was determined by \( \sqrt{\frac{S^2}{N}} \). Critical difference between treatments were calculated by the following formula:

\[ \text{C.D.} = \text{S.E.} \times \sqrt{2} \times t \]

In all these calculations \( S^2 \) stands for variance, \( S \) for standard deviation, \( X \) for observations (i.e. growth in mg), \( N \) for number of observations and 't' for table values of 't' at \( N - 1 \) degrees of freedom at 5% or 1% levels of significance.

After calculating C.D. the significance of observations was determined. If the observed value went beyond arithmetic mean ± C.D. (at 5% or 1%), then it was supposed to be significant. The significance at 5% is noted by one (*) and at 1% by two (**) asterisks. The treatments are considered to be significant and highly significant respectively.