Material and Methods
PART - I

ISOLATION OF ASPERGILLI

1) Collection of seed samples:

The methods described by Neergaard (1973) has been adopted for the collection of seed samples. Accordingly, seed samples were collected from field, store houses and market places. A composite sample was prepared by mixing the individual samples together, preserved in cloth bags at room temperature during the studies.

2) Detection of seed mycoflora:

The procedure for blotter test and agar plate methods was followed as described by International Seed Testing Association, ISTA (1966). De Tempe (1970), Neergaard (1973) and Agarwal (1976).

1) Blotter test method:

A pair of white blotter papers of 8.5 cm diameter was jointly soaked in sterile distilled water, placed in presterilized corning Petriplates of 10 cm diameter. Ten seeds per plate were placed at equal distance on the moist blotters. One hundred seeds were tested for each treatment. The plates were incubated at $25 \pm 2^\circ C$ under diurnal condition.
On 7th day the seeds were examined under stereoscopic microscope for the preliminary determination of *Aspergilli*. Identification and further confirmation of different species of *Aspergillus* and other fungi occurred on seeds was made by preparing slides of the fungal growth and observing under compound microscope.

11) **Agar plate method:**

In this method, pre-sterilized corning glass Petri-plates of 10 cm diameter were poured with 25 ml of autoclaved potato dextrose agar (PDA) medium. On cooling the medium, 10 seeds per plate were equispaced aspectically, incubation condition and other details were same as described for the blotter test method.

In order to isolate only internal seed mycoflora, seeds were pretreated with 0.1% solution of mercuric chloride for 1 minutes, subsequently thoroughly washed twice with sterile distilled water and placed on agar plates. Seeds without any such pretreatment were employed for the total seed mycoflora (control).

3) **Composition of media used in isolation studies:**

Impact of different agar media on percent incidence of *Aspergillus* species was studied by using following types of media:
1) **Potato Dextrose Agar (PDA)**:

200 g peeled potatoes were boiled until soft and passed through muslin cloth, 20 g of dextrose was added to it and final solution was made upto 1000 ml. In this 20 g of Agar agar was added, pH of the medium was adjusted to 5.6.

ii) **Czepek's Dextrose Agar (CZA)**:

Sucrose 15 g, NaN$\text{O}_3$ 2 g, KH$_2$PO$_4$ 0.1 g, MgSO$_4$ 0.5 g, Agar 20 g and distilled water 1000 ml, pH 5.6 was adjusted.

iii) **Glucose Nitrate Agar (GNA)**:

Glucose 10 g, KNO$_3$ 2.5 g, KH$_2$PO$_4$ 1 g, MgSO$_4$ 0.5 g, Agar 20 g and distilled water 1000 ml, pH 5.6 was adjusted.

iv) **Seed extract Agar (SEA)**:

10 g seeds of different crops were boiled separately in 200 ml distilled water for 20–30 minutes. The extract was filtered through two layers of muslin cloth. The final volume of the extract was obtained to 1000 ml with distilled water and then 15 g of agar was added to it. The pH was maintained at 5.6.
PART - II

BIODETERIORATION OF SEEDS:

The seeds of groundnut, bajra and blackgram and neem were surface sterilized separately with 0.1% HgCl₂ solution and washed twice with sterile distilled water. Excess water was discarded, the seeds were distributed into pre-sterilized conical flasks (25 g/flasks) and were inoculated separately with 2 ml spore suspension of different species of Aspergillus. The flasks were incubated at room temperature for 15 days and were harvested for recording chemical changes in the seeds due to the fungi. For which the seeds were thoroughly washed under running tap water in order to remove mycelial growth from their surfaces. Subsequently the seeds were dried at 60°C for 48 hours and crushed into fine powder for the estimation of different chemicals. For the control, seeds were incubated in a similar manner but without inoculating the spore suspension of Aspergilli.

1) Estimation of ash:

One g of seed powder was placed in a previously weighed crucible and it was subjected to heating on a hot plate for about 30 minute till the sample was sufficiently turned black. Then it was placed in muffle furnace pre-heated to 600°C for 2 hours with automatic control. Crucibles
were transferred directly to dessicator, cooled and weighed immediately. Weight of ash was obtained and reported as percent ash.

11) **Estimation of protein**:

Estimation of crude protein was made by microKjeldahl method (A.O.A.C. 1960) 300 mg dry powder of seeds was placed in 50 ml MicroKjeldahl flask. 60 mg catalyst and 7.5 ml H$_2$SO$_4$ were added in the flask. The flasks were heated for 6-8 hours (Digestion). After, this on cooling the flasks, the digest was diluted to 50 ml in a volumetric flask. 5 ml of the aliquot was introduced in Markham's distillation unit through the side tube funnel to which glass stopper was fitted. 10 ml of 40 % NaOH was allowed to run it into the digest. NH$_3$ liberated was collected in 50 ml conical flasks containing ml of 2 % Boric acid with indicator and the distillation was titrated against 0.035 NHCl till end point was achieved. The crude protein was calculated as percent N x 6.25 = crude protein.

111) **Estimation of fat**:

Fat estimation was done by ether extraction method. One g of seed powder was placed in a thimbel, placed in an extractor at 40-45°C for one hour. Then the solvent
ether along with extracted seed fat was poured in a pre-
weighed disc. Ether was evaporated under fan and residue
was dried over night in an oven at 60°C. The dish was
immediately transferred in a dessicator. On cooling, it
was adjusted and the amount of fat extracted was reported
as percent crude fat.

iv) Estimation of starch:

It was estimated by anthrone reagent method (A.O.A.C.
1966), 100 g seed powder was mixed with 5 ml of distilled
water, 6.5 ml 52% perchloric acid was added to it in order
to dissolve the total starch. The mixture was placed at
0°C for 20 minutes, centrifuged and retained the supertant
extract. The residue was repeatedly extracted with the
addition of fresh perchloric acid. The combined extract
was diluted to 100 ml. The diluted extract (0.5 ml) was
taken in a test tube and to it 4.5 ml distilled water was
added. 10 ml cold anthrone sulfuric acid reagent was mixed
with 5 ml above extract in an ice-bath heated for 8 minutes
at 100°C and cooled rapidly to room temperature. Then the
O.D. was measured at 630 mm, calibration curve was pre-
pared by using glucose solution. Starch content was cal-
culated by multiplying by 0.9 the equivalent.
Anthrone sulphuric acid reagent - 200 mg anthrone was dissolved in 100 ml of cold 95 % H₂SO₄ and stored at 0°C.

**STUDIES ON HYDROLYTIC ENZYMES:**

1) **Amylase**
   a) **Production**

Production of amylase was studied by growing the species of *Aspergillus* on liquid medium containing starch, 1 %; KNO₃, 0.25 %, KH₂PO₄, 0.1 % and MgSO₄ 7H₂O, 0.05 %. pH of the medium was adjusted to 5.5. Twenty five ml of the medium was poured in 100 ml conical flasks, autoclaved and inoculated separately with 1 ml spore suspension of the fungi which were maintained on PDA slants for 7 days. The flasks were incubated for 6 days at 25±2°C with diurnal periodicity of light. On 7th day the flasks were harvested by filtering the contents through Whatman filter No.1. The filtrates were collected in presterilized bottles and termed as crude enzyme preparation.

b) **Assay (Cup-plate method)**

Determination of amylase activity was done with the help of cup-plate method which was adopted by Singh and Saksena (1982), where 25 ml of starch agar assay medium.
(soluble starch 10 g, Na$_2$HPO$_4$ – 2.84 g, NaCl – 0.35 g, agar agar 20 g, Dlw – 1000 ml and at pH 6.9). 15 ml of the medium were poured in each Petriplate. On solidifying the medium, a cavity (8 mm diameter) was made in the centre with the help of a cork borer (No.4) and was filled with 1 ml culture filtrate (crude enzyme preparation). The plates were incubated at 28°C for 24 hours then they were flooded with Lugol's iodine solution as an indicator. A clear, non blue, circular zone was obtained surrounding the central cavity. The diameter which was measured (mm) as the amylase activity zone. Similar procedure was followed for the control except pouring of autoclaved culture filtrate in the central cavity instead of the active enzyme.

2) Lipase:

a) Production:

Production of lipase was studied by growing the Aspergillus species on liquid medium at pH 5.6 containing oil 10 g, KNO$_3$, 2.5 g, KH$_2$PO$_4$, 1 g, MgSO$_4$, 0.5 g and distilled water 1000 ml.

Further details are similar to that described for studies on amylase production. The culture filtrates termed as crude lipase preparations.
b) Assay (Cup-plate method):

Determination of lipase activity was done with the help of cup-plate method which was adopted by Sierra (1957). The medium contains Difco peptone 10 g, NaCl, 5 g, CaCl$_2$.2H$_2$O, 0.1 g and agar agar 20 g per litre and 10 ml lipid substrate sorbiton monolaurate (Tween - 20) presterilized was added to it. pH of the medium was adjusted to 6.0. 15 ml of the medium were poured in each Petriplate, on solidifying the medium, (No. 4) a cavity (8 mm diameter) was made in the centre with the help of cork borer and was filled with 0.1 ml of enzyme preparation in triplicates. They were incubated at 28°C after 24 hours, a clear circular zones was observed surrounding to the central cavity. Diameter of the zone was measured (mm) as lipase activity. Similar procedure was followed for the control except the pouring of autoclaved enzyme preparation in the central cavity instead of the active enzyme.

3) Protease:

a) Production:

The fungi were grown on liquid medium containing glucose 10 g, KH$_2$PO$_4$ 1.0 g, MgSO$_4$7 , casein hydrolysate 5 g in 1000 ml distilled water. pH was adjusted at 5.6.
Further details are similar to that described for amylase production. The culture filtrates were termed as crude protease preparations.

b) Assay (Cup-plate method):

Determination of protease activity was done with the cup-plate method adopted by Rao and Mukherjee (1990). A basal medium was prepared by adding 4% solution of gelatin in nutrient agar. The pH of the medium was adjusted to 6.5. 15 ml of the medium were poured in Petriplate. On solidification of the medium a cavity was prepared in centre of the plate with the help of presterilized cork borer (8 mm dia). The cavity was filled with 1 ml culture filtrate (crude protease) and incubated at room temperature. After 24 hours, the plates were flooded with saturated solution of 15% HgCl₂ in 7 M HCl. A clear zone appeared around the cavity after 10 minutes. Diameter of the zone considered proportional to the protease activity while, non-appearance of zone considered absence of protease in the culture filtrate.

4) Cellulase:

a) Production:

The fungi were grown on liquid medium containing 1% cellulosic substrate (CMC), 0.25% KNO₃, 0.1% KH₂PO₄,
0.05% \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \). pH of the medium was adjusted to 5.5. Further procedure is similar to that described for amylase production. The culture filtrates termed as crude cellulase preparations.

b) **Assay (Cup plate method):**

As per the procedure described by Dingle *et al.* (1953) and Szecsi (1969) 25 ml of assay medium (CMC agar) containing 1% CMC + 2% Difco agar was poured in Petridishes (9 cm diameter) and allowed to solidify.

Then in the centre a 8 mm diameter cup (cavity) was made with sterilized cork borer (No.4). The cup was filled with 0.1 ml culture filtrate and incubated at room temperature for 48 hours. The activity zones were developed flooding the plates with 3% lead acetate solution (10-15 ml/dish) and incubated for 20-40 minutes milky white coloured activity zone was clearly seen around the cup after removing lead acetate solution by running distilled water in the plates. Diameter of the zone was measured as cellulase activity. Similar procedure was followed for the control except the pouring of autoclaved culture filtrate in the central cavity instead of the active enzyme.
5) **Pectinase** :

a) **Production** :

The fungi were grown on liquid medium containing pectin 1.0 %, KNO₃ 0.25 %, KH₂PO₄ 0.1 % and MgSO₄ 0.05 % pH. The medium was adjusted at appropriate. Further procedure is similar to that described for amylase production. The culture filtrates termed as crude pectinase preparations.

b) **Assay (Viscometry method)** :

Pectin was used as substrates. The substrate enzyme mixture had the following compositions:

- 2 % pectin - 5 ml
- Active crude culture filtrate at
- Appropriate pH - 3 ml
- Distilled water - 2 ml

Viscosity measurements were carried out with an Ostwald viscometer with 9 seconds as the flow rate for water.

The tube containing substrate solution and the viscometer were thoroughly cleaned and dried before hand, suspended in the vertical position inside a water bath at 25°C and both were left for a few minutes to acquire the temperature of the bath.
Three ml of enzyme solution was then added to the substrate and simultaneously a stop watch started. The substrate enzyme mixture was stirred quickly and then it was transferred to the viscometer. Viscosity reading were taken at 0, 2, 3, 10, 20 and 30 minutes after adding the enzyme solution.

Viscosity represented as the time of the flow in seconds of the reaction mixture through the capillary of the viscometer was plotted against time in minutes. In some 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:

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

Total possible loss of viscosity = \( V_o - V_w \)
Viscosity loss due to the action of enzyme = \( V_o - V_t \)
\[ \% \text{Viscosity loss due to the action enzyme} = \frac{V_o - V_t}{V_o - V_w} \times 100 \]
The substrates were used to make viscosity/time measurements and the percentage viscosity loss of the substrates was calculated with the formula given above.

STUDIES ON TOXINS:

1) Phytotoxins:
   a) Production:

   Aspergillus species were grown on liquid medium containing glucose 10 g, KNO$_3$ 2.5 g, KH$_2$PO$_4$ 1 g, MgSO$_4$ 0.5 g and distilled water 1000 ml. 25 ml of the medium was added in 100 ml conical flasks and autoclaved at 15 lb pressure for 15 minutes. Then the flasks on cooling were inoculated separately with 1 ml standard spore suspension of the fungi prepared from seven day old cultures grown on PDA slants. The flasks were incubated at 25±2°C for 8 days and were harvested by filtering their contents through Whatman filter paper No.1. The filtrates were collected in presterilized culture bottles and termed as crude toxin preparations. Crude toxin preparation were tested for their toxicity.

   b) Assay methods:
      1) Seed germination:

      100 seeds of test crop were soaked in culture filtrates (toxin) for 24 hours. They were then placed on
moist blotters in petriplates. Seeds soaked in freshly prepared liquid medium served the control. Percentage germination, root and shoot elongation were recorded over a period of five days.

2) **Wilting of shoot cuttings:**

Shoot cuttings (6 inches long from the tip) of *Medicago sativa* L. grown in the field were used for the test. 5 ml culture filtrate was taken in a glass vial in which the shoot cutting was dipped to the level of culture filtrate and incubated for 24 hours at room temperature. Shoot cuttings kept in freshly prepared sterile medium served the control. The wilting symptoms caused due to the culture filtrates were recorded as follows:

1) **Water soaked lesions on lvs,**
2) **tip drying**
3) **shoot wilting,**
4) **shoot necrosis,**
5) **leaf curl.**

3) **Leaf necrosis :**

For this, freshly collected leaves of *Medicago sativa* L. were employed. The leaves were washed with
sterile distilled water and were placed on moist filter paper in Petridishes. A drop of 0.01 ml culture filtrate was placed on the surface of leaf by making a minor injury to it. Leaves with 0.01 ml sterile fresh medium served as control. After 24 hours incubation at room temperature the development of necrotic, chlorotic or water soaked lesions were recorded.

2) Aflatoxin :

a) Production :

Thirteen isolates of *A. flavus* were screened for the production of aflatoxins. For this liquid medium namely SMKy medium (Diener and Davis, 1966) containing Sucrose 20 g, KNO₃ 3.0 g, MgSO₄·7H₂O 0.5 g, yeast extract 7 g, distilled water 1000 ml employed. Each medium of 50 ml aliquots was taken in 250 ml capacity Erlenmeyer flasks and inoculated with 0.25 ml of a spore suspension prepared from 8 day old culture of each of the isolate grown on PDA slants. Each treatment was carried out in triplicates. The inoculated flasks were incubated for 7 days in a stationary condition at 25+1°C. After incubation, the contents of each flask were filtered through previously weighed Whatman filter paper No.1
The culture filtrates were stored at 4°C to be tested for the presence of aflatoxins by thin layer chromatography.

Assay (TLC) (Chromatographic analysis):

Aflatoxin production was determined by thin layer chromatography. Analyses were performed on 5 ml samples of filtrates which were extracted with 10 ml of chloroform. The chloroform extracts were evaporated to 2 ml. Aflatoxins were determined qualitatively in every chloroform extracts by spotting samples on thin layer chromatoplates (20 x 20 cm), 0.3 mm thickness of silica gel which has been activated at 120°C for 2 hrs. Proper controls of aflatoxin B₁ standard obtained were also spotted. Plates were developed with solvent system of 2 % methanol in chloroform in a chamber. Plates were examined in dark under long wave UV lamp. Formation of aflatoxin B₁ was confirmed in the chloroform extracts of culture filtrates which yielded spots identical to that of the standard.

Extraction method for Artificially infected seeds:

100 g of A. flavus infected seed samples were stored
at saturated atmosphere to induce mould growth for 8 days at (27-29°C). At the end of incubation period the samples were oven dried at 60°C and finely ground. 50 g of finely powdered samples were weighed into waring blender. 200 ml of extraction solvent (Acetonitrile - 4 % KCl : 90 + 10) or (water-acetonitrile 100:50) were added and blend at high speed for 1-2 minute. 100 ml of supernatants were filtered through filter paper into graduated cylinder and transferred into 500 ml separatory funnel fitted with Teflon stop cock. Samples were defatated by extracting twice with 50 ml defatting solvent.

Extraction of aflatoxin:

50 ml of chloroform was added to above filtrate. The filtrate was extracted for 30 minute. After the layer have separated chloroform layer filtered into 100 ml Erlenmey flask and Aqueous layer was extracted twice with 50 ml of chloroform. The chloroform extracts was then passed through anhydrous Na₂SO₄ bed to remove moisture and evaporated to dryness and redisolved in 1 ml of chloroform for the detection and estimation of aflatoxin by TLC method.