MATERIALS AND METHODS

The target organism *Aspergillus umbrosus* (Bainier and Sartory; IMI No. 279833), has exhibited morphological abnormalities in laboratory culture. It was characterized by a very slow rate of growth, production of more sterile hyphal mass and considerably deformed conidial heads. In solid cultures there was good elaboration of brown to dark brown pigment, in the form of a halo around the colony (Plate - 1). It possessed a sufficiently high antagonistic potential towards a number of fungi (Kulshrestha and Ali; 1986).

A study was undertaken to investigate this organism, with respect to its physiology of growth and antibiotic production, and to evaluate it for some practical application. The objective was to obtain better vegetative growth, good sporulation and good amount of antibiotic production. The experiments carried out for the objectives include determinations of the effects of nutritional and cultural factors as follows:

1. **Effect of different culture media** :
   The media taken were:

   A. **Synthetic media** :
   
   i. **Glucose - Asparagine** (Cole, 1956)
   ii. **Asthana and Hawker's** :

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>KNO₃</td>
<td>3.5 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.75 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
iii. **Sabouraud's:**

- Maltose: 40.0 g
- Peptone: 10.0 g
- Distilled water: 1000 ml

iv. **Cohn's:**

- $\text{KH}_2\text{PO}_4$: 5.0 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g
- Ammonium tartarate: 10.0 g
- KCl: 0.5 g
- Distilled water: 1000 ml

v. **Martin's:**

- Dextrose: 10.0 g
- Peptone: 5.0 g
- $\text{KH}_2\text{PO}_4$: 1.0 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g
- Distilled water: 1000 ml

vi. **Cole's tartarate:**

- Glucose: 20.0 g
- Ammonium tartarate: 10.0 g
- $\text{KH}_2\text{PO}_4$: 1.0 g
- Distilled water: 1000 ml

vii. **Pferrer's:**

- Ammonium tartarate: 10.0 g
- $\text{KH}_2\text{PO}_4$: 5.0 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 2.5 g
- Sucrose: 50.0 g
- $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: Trace
- Distilled water: 1000 ml


vi: i **Currie's:**
- Ammonium nitrate: 2.0 g
- \( \text{KH}_2\text{PO}_4 \): 0.75 g
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \): 0.25 g
- Sucrose: 125.0 g
- Distilled water: 1000 ml

ix **Richard's:**
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \): 0.25 g
- \( \text{KH}_2\text{PO}_4 \): 5.0 g
- \( \text{KNO}_3 \): 10.0 g
- Sucrose: 50.0 g
- Ferric chloride: Trace
- Distilled water: 1000 ml

### B. Natural substrate media:

i. **Brown's Synthetic potato** (Brown, 1925)

ii. **Potato - Dextrose - Peptone** (Koch, 1957)

iii. **Peptone - Glucose** (Johnson, 1957)

iv. **Maltose Peptone:**
- Maltose: 1.4 g
- Peptone: 50.0 g
- Distilled water: 1000 ml

v. **Starch casein:**
- Soluble starch: 10.0 g
- Casein: 0.3 g
- \( \text{K}_2\text{HPO}_4 \): 2.0 g
- \( \text{KNO}_3 \): 2.0 g
- NaCl: 2.0 g
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \): 0.06 g
- CaCO\(_3\): 0.02 g
- \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \): 0.01 g
- Distilled water: 1000 ml
vi. **Potato Dextrose:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>250.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

In each case the fungus was grown in broth medium after being inoculated uniformly (a 4 mm disc of freshly grown colony per 25 ml medium) and incubated at 30±1°C for 12 to 15 days under aseptic conditions. Observations were taken for total fungal biomass by weight, sporulation by visual gradation and pigment production from colouration produced. Simultaneously, cultures were made on solid media for microscopic examination of the fungus in respect of the effect of valuable cultural conditions on its vegetative and reproductive morphology. Morphological observations have been presented in plates.

The effect of medium was studied in each case at 5 different pH values, i.e. 3.5, 4, 4.5, 5 and 5.5 to record suitability of pH in relation to a particular medium. The pH values were set before autoclaving.

2. **Effect of different carbon sources:**

The different Carbohydrates taken were; L-arabinose, D-galactose, D-maltose, D-mannitol, D-raffinose, D-sorbitol and D-xylose.

In each case the carbon source was taken in 3% w/v, and added to the basal Richard's Medium in place of sucrose. Cultural conditions and record of observations were the same as earlier.

3. **Effect of the concentration of the best carbon source:**

D-mannitol was found as the best carbon source for this fungus. The following concentrations 3, 5, 7.5, 10 and 12.5%; were taken in a higher order to determine its optimum effect on physiology of fungus.
4. **Effect of nitrogen source:**

To determine the effect of nitrogen source some of the amino acids were selected to replace the inorganic nitrogen ($\text{KNO}_3$) in Richard's medium before autoclaving. The amino acids taken were; $\text{L - arginine}$, $\text{L - aspartic acid}$, $\text{L - glutamic acid}$, $\text{L - methionine}$, $\text{L - tyrosine}$, $\text{L - tryptophan}$, $\text{L - valine}$ and $\text{DL - alanine}$. In each case the amino acids were added in 0.1% w/v concentration.

In another experimentation the potassium nitrate was taken either with $\text{L - arginine}$ or $\text{L - aspartic acid}$ as they provided best in the following variable proportions to determine the effect of organic and inorganic nitrogen source synergistically.

<table>
<thead>
<tr>
<th>% Concentration of $\text{KNO}_3$</th>
<th>% concentration of $\text{L-arginine}$ or $\text{L-aspartic acid}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>

5. **Effect of vitamins:**

For the following determinations the basal medium selected was Richard's that was modified as per the result of earlier experiments.
The composition of this medium was:-

D-mannitol 3.0%
KNO₃ 1.5%
L-arginine 0.5%
KH₂PO₄ 0.5%
MgSO₄·7H₂O 0.025%
FeCl₃ Trace
Distilled water 1000 ml

The water soluble vitamins taken were: nicotinic acid, folic acid, riboflavin and ascorbic acid. In each case the vitamins were added in 0.05% w/v concentration in modified Richard's medium after autoclaving. After the addition of vitamins, medium was sterilized by filtration through Millipore bacteriological filters (Pore size 0.45 μm, Sartorius, SM. 11306).

6. Effect of plant growth hormones:

The different plant growth hormones taken were; indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), napthyl acetic acid (NAA), gibberellic acid (GA), 6-benzyl adenine (BA) and kinetin. In each case the hormones were sterilized by filtration and added in the medium in 10, 15, 20 and 25 ppm concentrations after autoclaving.

7. Effect of metal ions:

To determine the effect of metal ions, eight metal salts; Cr₂(SO₄)₃, CoCl₂, NiCO₃, CuSO₄, Na₂MoO₄, ZnSO₄, FeSO₄ and SrCO₃ were taken. In each case five different concentrations; 100, 200, 300, 400 and 500 ppm were tried. The basal medium here again was Richard's modified medium and the salts were added before autoclaving.
8. **Effect of the temperature of incubation:**

The cultures were grown on the modified Richard's medium and then incubated at different temperatures, i.e.; 26, 30, 35, 40, 45 and 50°C for 12 - 15 days.

9. **Optimum incubation period for the best biomass:**

The cultures were grown on the modified Richard's medium, to determine the best period for optimum growth and antagonistic effect. Observations were taken after harvesting the culture and filtrate, from 6th day up to the 30th day at regular intervals of 2 days each.

10. **Effect of shaking:**

Cultures were grown under shaking in a "Lab line" (U.S.A.) Rotatory Shaker Incubator in modified Richard's broth medium at 30±1°C. Data was recorded for growth, sporulations and pigmentation from 6th days onwards up to the 14th day by harvesting on alternate days. Simultaneously, still cultures in broth medium were also taken for comparison.

**Studies on Antibiosis:**

The **antibiosis** of *A. umbrosus* has been evaluated by

1. direct measurement of antagonism in co-culture and

2. analysis of culture filtrate for the presence of active principle (metabolite)

**1. Antagonism in Co-culture:**

This has been determined by employing various methods, viz.
i. Co-plating of test and antagonist.
ii. Using spore suspension of the test organism against the antagonist's colony.
iii. Over-laying of antagonist on plated test organism, and
iv. Employing the food-poison technique of Nene and Thapliyal (1965).

The test organisms against which the antagonism of A. umbrosus was determined were:


Bacteria: Escherichia coli (gram -ve), Bacillus subtilis (gram +ve) and Streptomyces coelicolor.

i. Co-plating Method:

The medium used in this method was potato-dextrose agar, at pH 4.5. Both, the Aspergillus umbrosus and test organism were inoculated in the same plate, at a little distance from each other and incubated at 30±1°C. The size of the zone of inhibition (mm) was recorded after sufficient growth.

ii. Spore suspension method:

The medium used for fungi was potato-dextrose-agar and nutrient agar for the bacteria.

In this method spore suspension of test organism was prepared in sterilized distilled water. This spore suspension of the test organism was added to the molten sterilized medium
and plated. After solidification of the medium, mycelial discs of *A. umbrosus* were also plated in each of the seeded plates and incubated. Data of growth inhibition if any in respect of the test organism was recorded after 10 days of incubation at 30±1°C.

iii. Over-lay method:

Here the medium incubated with the spore suspension of the test organism was poured over a well grown culture of *A. umbrosus* and both were simultaneously incubated for 10-12 days. Formation of clear zone over the *A. umbrosus* colony in the seeded layer was observed and recorded as measure of activity.

In yet another way the *A. umbrosus* was over layed on the seeded plate of the test organism and incubated. Data was similarly taken.

iv. Food poison technique.

The method described by Nene and Thapliyal (1965) was followed here. The crude culture filtrate and its ethyl acetate fraction were taken and added to the culture medium after autoclaving. The test organisms were then inoculated on the plates containing the culture filtrate or its fraction and incubated. Record of growth was taken as measure of activity.

2. Analysis of the Culture filtrate and detection of the active antibiotic principle:

The culture of the fungus was grown in 3 litres Haffkin's flasks on modified Richard's broth medium repeated to obtain about 4 litres of the crude culture filtrate, the cell free culture filtrate was thoroughly mixed with 1 litre of ethyl-acetate to extract fractions that are soluble in it.
After keeping the immiscible mixture over night, the ethyl acetate and water fractions were separated, concentrated under vacuo, and used for column chromatography to fractionate compounds that may possess antibiotic property.

**Chromatographic Separation and elution:**

The prepared sample in ethyl acetate was passed through columns of Silica gel and Alumina. Fractions were eluted with 1. Ethyl-acetate, 2. Acetone, 3. Acetone:Ethanol (1:1 v/v), 4. Ethanol, 5. Ethanol:Water (1:1: v/v), and 6. water in the same sequence. The eluents were then used for UV-spectrophotometric analysis to have absorption spectrum by scanning the compound over 225-325 nm.

The fungal antibiotics Penicillin and Cephalosporin were also scanned on UV-Spectrophotometer (Varian, DMS. 100) between 225-325 nm to serve as reference. The UV-scans (absorption spectrum) with peaks have been compared to understand the possible nature of the active antibiotic compounds extractable in ethyl acetate from the crude culture filtrate of *A. umbrosus*. 