Chapter-III

Materials and Method
MATERIALS AND METHODS

Many edible mushrooms are cultivated commercially throughout the world as vegetable crops. Therefore, two species of *Pleurotus* and one species of *Agaricus* were selected as edible mushrooms for the present study. The former was found to be most suitable in Bundelkhand region of U.P. due to its cheap cultivation technology, easy growth on various wastes under varying climatic conditions at temperature ranging from 20-30°C. *Agaricus bisporus* was selected due to its popularization and demand in public.

The experimental place, Department of Botany, Pt. Jawaharlal Nehru P.G. College Banda. The climate is typically monsoonal with high and low temperatures in summer (March-June), and winter (November-February) respectively with a heavy rainfall in the rainy season (June -September). The prevailing climatic condition of the year 2004-05 is presented in Appendix I. The materials used and the methods followed for the present investigation are described below.

1. MATERIALS

(a) Cultures of edible mushrooms :-

The cultures of *Agaricus bisporus*, *Pleurotus sajor-caju* and *Pleurotus florida* were procured from Chandra Shekhar Agriculture
University, Kanpur (U.P.).

(b) Substrates for cultivation :-

Various agro-wastes and other substrates namely wheat straw, paddy straw, sugarcane bagasse, maize stalks mustard stem, linseed stem were evaluated for cultivation of various Pleurotus spp. while wheat straw was used as a base material for cultivation of button mushroom. Wheat bran, flour of cereals and oil cakes were used as supplements. Chicken manure was used in compost preparation whereas farm yard manure with garden soil was used as casing material. All these substrates and raw materials were obtained from local sources.

Standard chemical fertilizers and commercial grade gypsum were obtained from commercial outlet. Polythene bags as containers for cultivation and half litre glucose bottles for spawn preparation, were also obtained from local commercial outlet. Analytical reagent chemicals were used in cultural and laboratory work. Borosil glasswares were used throughout the study.

(c) Culture media :-

Different culture media are mentioned below which were used during the the course of the present study:
1. **Malt-extract Agar medium**
   
   Malt extract 20.0 g  
   Malic acid 5.0 g  
   Agar 20.0 g  
   Water (distilled) 1000 ml  
   pH 5.6  

   This medium was selected for sub-culturing of the pure cultures of edible mushrooms.

2. **Potato-Dextrose agar (PDA) medium** (Riker and Riker, 1936)
   
   Potato (peeled and sliced) 200.0 g  
   Dextrose 20.0 g  
   Agar 18.0 g  
   Water (distilled) 1000 ml  
   Streptomycin sulphate 30 \( \mu \)g/ml  
   pH 5.6  

3. **Czapek-Dox + Yeast extract (CDA) medium** (Raper and Thom, 1949).
   
   Mg\( \text{SO}_4 \cdot 7\text{H}_2\text{O} \) 0.5 g  
   Kcl 1.0 g  
   NaNO\( _3 \) 2.0 g  
   KH\( _2\)PO\( _4 \) 1.0 g  
   FeSO\( _4 \) 0.066 g  
   Sucrose 30.0 g  
   Yeast extract 2.0 g  
   Agar 18.0 g  
   Water (Distilled) 1000 ml  
   pH 5.6  

*This medium was used for isolation of fungi from the compost.*
4. *Peptone-Dextrose agar medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Water (Distilled)</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>30 μg/ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>5.6</td>
</tr>
</tbody>
</table>

*This medium was used for isolation of fungi from the compost.

5. *Nutrient agar medium (Difco Manual, 1953)*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
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<tr>
<td>Peptone</td>
<td>5.0 g</td>
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<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>18.0 g</td>
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<tr>
<td>Water (distilled)</td>
<td>1000 ml</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.3</td>
</tr>
</tbody>
</table>

The antibiotics where required were added only after autoclaving.

2. METHODS

2.1. Effect of environmental factors :-

Various environmental factors like, temperature, relative humidity, light and aeration governing the fructification of *Pleurotus* sp. were considered in the present investigation. Three replicates for each treatment were maintained throughout the study of each environmental parameter.
(a) Temperature :-

The completely colonized bags were subjected to different temperatures ranging from 15-35° C. Other factors were maintained constant as far as possible. The average yield in gram per 500 gram dry wheat straw substrate was recorded.

(b) Relative humidity :-

To study the different levels of relative humidities on yield of different species of Pleurotus, different ranges of humidities (65-90%) were maintained in controlled humidity chamber. Humidity was recorded with the help of a hygrometer. The completely colonized bags were exposed to the varying humidities for corp production. The yield was recorded in gram per 500 gram dry wheat straw substrate.

(c) Aeration :-

After complete spawn run, the substrate bags were subjected to different periods of aeration viz., one hour, two hours, three hours, four hours aeration in 24 hours as well as without aeration throughout the cropping period. The yield was recorded in gram per 500 gram dry wheat straw substrate.

(d) Light :-

The spawn run bags were exposed to optimum growing condition, varying only the light exposures from one hour light, alternate light and dark to continuous light with the help of Philips tube lights. The absolute darkness was maintained by covering the chamber with thick black cloth.
2.2. Spawn preparation :-

Wheat-grain spawn was prepared by the standard method (Muratal, 1973) for both Pleurotus sp. and Agaricus bisporus. Spawning was done by ‘through mixing’ procedure and the rate of spawning was kept between 0.5-1.0% which was followed throughout the study.

2.3. Pre-treatment of substrate for cultivation of Pleurotus sp:-

Pre-treatment of the substrate is essential process before spawning to reduce to kill the contaminants and other saprophytic microflora associated with straw which may otherwise hinder the proper growth and development of mushroom mycelium. Before pre-treatment, various agro-wastes were chopped off and cut into small pieces (2-4 cm) to facilitate proper wetting as well as to increase surface area. Chemical Sterilization Technique (CST) advocated by Vijay and Sohi (1987) was followed throughout the study for substrate treatment except in some specific cases which are mentioned at appropriate place in the text.

2.4. Compost preparation for cultivation of Agaricus bisporus :-

Agaricus bisporus needs well composted material for its growth and fructification. The compost was prepared by long method (Seth, 1975). The wheat straw was wetted overnight and the content was mixed thoroughly before stacking on 0-day. Moisture content on the day of stacking was maintained at 75 per cent. Gypsum at 30 kg per ton was added at
third turning. The turning schedule followed was as under.

<table>
<thead>
<tr>
<th>Day</th>
<th>Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1st turning</td>
</tr>
<tr>
<td>10</td>
<td>2nd turning</td>
</tr>
<tr>
<td>13</td>
<td>3rd turning</td>
</tr>
<tr>
<td>16</td>
<td>4th turning</td>
</tr>
<tr>
<td>19</td>
<td>5th turning</td>
</tr>
<tr>
<td>22</td>
<td>6th turning</td>
</tr>
<tr>
<td>25</td>
<td>7th turning</td>
</tr>
<tr>
<td>28</td>
<td>filling day</td>
</tr>
</tbody>
</table>

Before spawning, the temperature of compost was brought down at the room temperature (25°C)

2.5. Effect of duration of composting on spawn run and yield of *Agaricus. bisporus* :-

The compost samples were taken from the stack of compost on 8th, 12th, 16th, 20th, 24th and 28th day for the effect of duration of composting on spawn run and yield of A. *bisporus*. Ther equal amount of compost was filled in sterilized test tubes upto 3 cm below the mouth of the tube (size 15 cm x 2.5 cm) and 3-4 colonized wheat grains by A. *bisporus* were put over the compost in each test tube i.e top spawning. The tubes in 4 replicates were incubated at 25+2°C. The spawn run was measured from top to bottom for linear growth of mycelium after 3 weeks of incubation.

To study the yield response of the mushroom, 2 kg spawned compost (on fresh weight basis) was filled in each of 3
replicate polythene bags and were incubated at 24±2°C for 3 weeks there after the casing was done and the yield was recorded during cropping period for a month.

2.6. Supplementation of the compost :-

Supplements are materials added to compost at any time after the completion of composting for direct utilization by mushroom mycelium. Before application, all the supplements were treated with 5000 ppm formaldehyde and were kept as such in polythene bags for 24 hours. In case of Agaricus bisporus the rate of supplementation was kept 0.5%, 1% and 1.5% (on fresh weight basis of compost) and supplements were applied to the compost at the time of spawning and casing as required.

In case of Pleurotus species, supplementation was done at the rate of 1% (on wet weight basis) and was applied to the pre-treated substrate before spawning.

2.7. Spawn run :-

After spawning, 500g substrate per bag on dry weight basis for Pleurotus sp. and 5 kg compost per bag on fresh weight basis for Agaricus bisporus, were filled in polythene bags. The mouth of the bags was tied up with thread. These bags were kept in cropping room at 25°C for spawn run. Other optimum conditions were maintained as necessary for proper spawn run.

2.8. Casing :-

Casing is essential for pinhead formation in case of
*Agaricus bisporus*. Casing material used for experiments was a mixture of farm yard manure (2 years old) and garden soil in 3 : 1 ratio, after treatment with formaldehyde (at 1 lit per m$^3$ of casing soil). Uniform 3-4 cm thick casing layer was put on top to spawn run compost bags. Care was taken to keep the casing layer wet by giving regular water spraying.

2.9. Cropping :-

For cropping of *Pleurotus* species a well ventilated cropping room with window and exhaust fan was used which were fumigated with 2% formaline before use. Desired temperature and humidity was maintained through frequent water spraying on walls and floors of room. The yield data of whole cropping period were recorded. The biological efficiency was worked out as the percentage weight of the mushroom produced on the dry weight basis of straw.

For cropping of *Agaricus bisporus* an air conditioned room and a well ventilated room with window and exhaust fan were used which were fumigated with 2% formaline before use. Under seasonal growing condition, the natural temperature before casing was 24+2°C whereas it was 15-21°C after a week of casing.

2.10. Sterilization :-

The culture media were sterilized by autoclaving at 121°C for 30 minutes. The glasswares were sterilized in an oven at 180°C for 2-4 hours. Wheat grains after boiling were sterilized at 121°C for on hour, for spawn preparation.
2.11. **Isolation of mycoflora from the compost** :-

Isolation of mycoflora, inhabiting during fermentation was done from the samples taken from the stack of compost (1.5×1×1 meter³ dimension). The samples were collected from upper surface (A) (10 cm deep from the upper surface), centre of the stack (B) and bottom (C) (10 cm above the ground floor) on each turning day just before the stack was broken to re-stack. Dilution plate technique was adapted to isolate the mycoflora. The temperature of respective zone was recorded at the time of samplings. Compost samples were shaken mechanically for 30 minutes before dilution and 10⁴ dilution was used for the purpose. The inoculated petri dishes were incubated at 25+2°C and 48+2°C for the growth of mesophilic (M) and thermophilic (T) fungi respectively. Triplicates were kept for all A, B and C. The petri dishes were examined after a week for record of fungi.

Percent occurrence of the individual fungi isolated by dilution plate technique, was estimated using the formula given below:-

\[
\text{% occurrence} = \frac{\text{Average number of total colonies of a species per plate}}{\text{Average number of total colonies of all the species per plate}} \times 100
\]

Four categories of occurrence were recognized, each with a quantitative significance as under:

0-25%, rare; 26-50%, frequent; 51-75%, common; and 76-100% dominant.
2.12. Organic carbon and nitrogen estimation:

For this purpose the compost samples were taken from all the turnings. The total organic carbon of the compost samples was determined by Walkley and Black method as outlined by Jackson (1985) and the total nitrogen was determined by micro-kjeldahl method as described in AOAC (1960).

2.13. Moisture estimation of compost: -

The moisture percentage of the ready compost was determined by taking known quantity of fresh compost from different zones and mixing them in one sample, dried in an oven at 60°C for 48-72 hours and weighed to a constant weight after drying. The loss in weight determined the moisture content.

2.14. Proximate composition of mushrooms:

Proximate analysis of various constituents like moisture, fat, carbohydrate, fibre, ash and nitrogen was done by following standard analysis according to Association of Official Analytica Chemists (19 %). Digestible crude protein was determined by multiplying N x 4.38 (Crisan and Sands, 1978).

Mineral composition of the sporophore was determined by digesting the samples with diacid mixture (HNO₃ : HClO₄) in the ratio of
4 : 1 (v/v). For analysis of K, Mg, Na, Ca, Fe, Atomic Absorption Spectrophotometer-against desired standard was used while total Phosphorus was determined with the help of Spectronic 20 (Jackson, 1967).

2.15. Identification:

The identification of wild mushroom flora encountered during survey and fungi isolated from compost samples was done chiefly on the basis of their morphological character with the help of the following literature.

1. Mushrooms (G.F. Atkinson, 1961)
4. Indian edible mushrooms (Purkayastha and Chandra, 1976)
5. Thermophilic fungi (Cooney and Emerson, 1964)
6. Dematiaceous hyponmycetes (Ellis, 1971)
7. Illustrated genera of imperfect fungi (Barnett and Hunter, 1972)
8. A manual of soil fungi (Gilman, 1975)

Besides the above mentioned literature, necessary help was taken from Dr. P.N. Singh, H.O.D. Mushroom Department C.S.A. University Kanpur, Dr. K.P.S. Kushwaha, Associate Director, Mushroom Department, GBUAT, Pantnagar for identification of the wild mushrooms.
2.16. Statistical analyses:

The data recorded were subjected to statistical analysis wherever required by applying Analysis of Variance (ANOVA) and Critical Difference (CD) and were calculated by standard methods (Goon et al., 1986). The details of the statistical inferences are given in Appendix-II.