CHAPTER II

MATERIAL AND METHODS

1. COLLECTION OF SAMPLES

The water samples were collected directly from the outlet where the combined factory effluent is released. Samples were also collected from the sedementation tanks which provided effluent water for irrigation. The water samples were collected in pre-sterilized, 250 ml glass-stopper bottles.

The soil samples were collected from the fields, irrigated by the waste water of the factory and from the marshy areas at the bases of canals carrying effluent. The soil samples were collected with the help of clean and sterile scalpels in sterilized Petri plates and corked specimen tubes.

The samples were stored in the refrigerator after being brought to the laboratory.

2. PHYSICAL EXAMINATION OF THE SAMPLES

Immediately after collection of the samples, pH was determined by using standard BDH pH paper. Total settleable solids of the water samples were determined by the method given in "The Standard Methods for Examination of

Water and Waste Water (APHA)" (Taras, 1971).

3. BIOLOGICAL EXAMINATION

Following techniques were used to study the mycoflora of the samples:

a. Bait Technique
   i. On straw
   ii. On filter paper
b. Serial dilution method using following media -
   i. PDA medium
   ii. Czapeks Dox medium
   iii. Waksmans' medium
   iv. Richards' medium
   v. Martin's rose bengal medium (D.P.A.)
c. Blotter technique - Larger debris pieces such as pieces of rags, paper, litter, etc., in samples from canals were incubated in moist chambers, i.e. Petri plates lined with wet blotters.

The fungi isolated by above techniques were purified and maintained on Czapeks Dox Agar or PDA Slants.

4. IDENTIFICATION OF THE FUNGI

The fungi obtained in pure culture were identified with the help of usual classification methods, referring to the standard monographs, other relevant literature and taxonomic keys.

Observations and camera lucida drawings were done under "Amplival Binocular Microscope".

Semi-permanent slides of some forms observed were prepared by "slide culture technique".

SLIDE CULTURE TECHNIQUE

A thin layer of sterilized, melted water agar medium was poured in sterile Petri plates. After the medium was solidified, approximately one square centimeter area was cut on it with the help of a sterile needle. A single block was then picked up by sterile needle and placed on flame-sterilized slide. The fungal spores or mycelial fragments were inoculated at the four corners of the agar block and covered with flame-sterilized cover-slip.

The slides were incubated in a moist chamber containing 20% glycerine (to avoid fogging). Periodically, microscopic observations were made to record the growth of the inoculated fungus. After the required growth was obtained the coverslip was removed and remounted in cotton-blue and lactophenol. Slides were sealed with wax.

5. SCREENING FOR CELLULOLYTIC ACTIVITY

All the isolated fungi were screened for their ability to degrade cellulose using simple method of measuring depth of clearing zone in a cellulose medium. Modified "Routella and Cowling medium" was used.

Composition of the medium:

$NH_4H_2PO_4$	2.00 g
KH_2PO_4	0.60 g
K_2HPO_4	0.40 g
$MgSO_4.7H_2O$	0.89 g
Yeast extract	0.50 g
Thiamine HCl	10.00 µg
Adenine	0.04 g
Adenosine	0.08 g
Acid swollen cellulose	2.50 g

(Cellulose powder was swollen in 85% O-phosphoric acid for 2 hrs at 4°C, washed in ice-cold distilled water by repeated suspending and decanting. Na_2CO_3 (1% w/v) was added and again washed in ice-cold distilled water until neutral).

Agar	17.00 g
Distilled water	1000.00 ml

8 - 10 ml of the above medium was poured in each test-tube, sterilized, cooled instantaneously and then inoculated. The depth of the clearing zone was measured (in mm) after 15 and 30 days.

6. SELECTION OF FIVE FUNGI FOR FURTHER STUDIES

Selection of following five fungi was done based upon their ability to degrade cellulose.

1. Aspergillus niger
2. Humicola grisea Traaen
3. Myceliophthora lutea Cost. var. macrospora var. nov.
4. Sporotrichum pruinosum Gil. & Abb.
5. Trichoderma viride Pers. ex Gray

These fungal forms were obtained in monosporous culture and maintained on Czapek-Dox medium slants.

To select appropriate medium giving maximum vegetative growth and sporulation the fungi were grown on following media:

1. Semi-synthetic media
   - i. Potato Dextrose Agar medium (P.D.A.)
   - ii. Corn Meal Agar medium (C.M.A.)
   - iii. Malt Agar medium (M.A.)
   - iv. M_2 Agar medium (M_2A)

2. Synthetic media
   - i. Czapek-Dox agar medium
   - ii. Richards' agar medium
   - iii. Martin's Rose Bengal agar medium (D.P.A.)
   - iv. Asthana-Hawker's agar medium
   - v. Sabouraud's agar medium
   - vi. Waksman's agar medium

7. EFFECT OF PHYSICAL FACTORS ON GROWTH AND SPORULATION

i) Effect of temperature on growth and sporulation

Equal amount of inoculum i.e. 3.0 mm disc from 8 days old culture of each fungus was inoculated in Petri plates containing sterile Czapek-Dox agar medium. Each fungus was inoculated in triplets and incubated in a temperature range of 0°C to 50°C. After 8 days incubation they were studied for colony diameter, colony morphology and sporulation.

ii) Effect of pH on growth and sporulation

30 ml of sterile, liquid basal medium was dispersed in 150 ml conical flasks. pH was adjusted in the range of pH 3 to pH 9 using digital pH meter (Philips). The flasks were inoculated in triplets, with 3 discs of the fungus of 3.0 mm diameter obtained from 8 days old colony. The flasks were incubated for 8 days. After 8 days dry weight of the mycelium was obtained by filtering the contents of each flask separately through a dry, pre-weighed filter paper (Whatman No. 42). pH of the medium after the incubation period was determined using the digital pH meter (Philips).

8. UITILIZATION OF CARBON

The ability of the selected fungi to utilize various carbon sources was studied using following carbon sources.

(1) Monosaccharides: Pentoses

i. D-Xylose ii. D-Arabinose

iii. D-Rhamnose

(2) Monosaccharides: Hexoses

i. D-Glucose ii. D-Fructose

iii. D-Mannose iv. D-Galactose

(3) Disaccharides:

i. Sucrose ii. Lactose

iii. Maltose

(4) Trisaccharide:

i. Raffinose

(5) Polysaccharides:

i. Starch ii. Inulin

iii. Dextrin iv. CMC

The quantity of the carbon sources replacing sucrose in the Czapek-Dox basal medium was calculated so as to ensure the same amount of available carbon as that from sucrose.

30 ml of liquid basal medium containing various carbon sources was dispersed in 150 ml conical flasks. The flasks were sterilized by intermittant sterilization on 3 successive days. The flasks were inoculated in triplicate, with 3 discs of the fungus. The discs (3.0 mm diam. each) were obtained from 8 days old colony of the fungus. The flasks were incubated for 8 days. Dry weight of the mycelium was determined after 8 days.

Enzyme activity of the culture filtrate with reference to cellulases was studied using the "Loss in Viscocity" method.

9. DETERMINATION OF CELLULOLYTIC ACTIVITY

30 ml of Czapek Dox basal medium with 0.5% CMC as carbon source was dispersed in 150 ml conical flasks. The flasks were inoculated in triplicate, with 3 discs (3.0 mm diam.) for each fungus from 8 days old colonies. The flasks were incubated for 8 days. After incubation the contents of the flasks were filtered. The filtrate was centrifuged at 4000 rpm for ½ hour and used as crude enzyme to determine cellulolytic activity by following methods:

i. Viscometric method
ii. Estimation of reducing sugars
iii. Cup plate method.

i. Viscometric method

Reagents

1. Carboxymethyl cellulose (CMC) solution:

0.5 g of CMC was dissolved in 100 ml of sodium acetate-acetic acid buffer at pH 5.2 at 50 - 60°C. The solution was filtered through Whatman filter paper No. 42 and stored at 4°C.

2. Sodium acetate - acetic acid buffer, pH 5.2.

Method: The cell free culture filtrate obtained by filtration and centrifugation was used as the crude enzyme.

4 ml of CMC solution and 2 ml of crude enzyme were mixed in a viscometer kept in a water bath at 30°C. Efflux time of the mixture at fixed interval was determined. Per cent loss in viscocity of CMC was calculated using the following formula -

$$V = \frac{T_o - T}{T_o - T_w} \times 100$$

where - V = per cent loss in viscocity

T_o = flow time in sec. at zero time

T = flow time in sec. of the reaction mixture at time T

T_w = flow time in sec. of distilled water.

ii. Estimation of reducing sugars (Dinitrosalicylic acid method)

Reagents

1. Dinitrosalicylic acid (DNS) reagent: 1 g DNS, 0.2 g crystalline phenol and 0.5 g sodium sulphite was dissolved simultaneously in 100 ml of 1% solution of NaOH by stirring. The reagent was stored at 4°C.

2. 40% solution of Rochelle salt (Sodium - Potassium tartarate).

Method: 3 ml of culture filtrate was mixed with 3 ml of DNS reagent in a test tube. The mixture was heated for 5 minutes in a boiling water bath. After the development of colour 1 ml of 40% Rochelle salt solution was added when the contents were still warm. Tubes were cooled under running tap. Absorbance of the solution was measured at 575 nm, on Beckmann's spectrophotometer. Amount of reducing sugar was calculated using standards prepared from glucose.

iii. Cup-Plate Method

25 ml of melted CMC-agar (1% CMC + 2% agar) medium was poured in a Petri plate and allowed to solidify. Then in the center a 8.0 mm diameter cavity (cup) was made with a sterilized No. 4 cork-borer. The cup was filled with 0.1 ml culture filtrate and incubated at room temperature for 8 days. The activity zones were developed with 3% lead-acetate solution (10 - 15 ml/dish). Milky white activity zones were seen after removing lead-acetate with distilled water. Diameter of the zone was measured which is directly proportional to the cellulase activity.

10. FACTORS AFFECTING THE DEGRADATION OF CELLULOSE (CMC / filter paper)

i. Effect of temperature on cellulolytic activity

30 ml of basal medium (without sucrose) was dispersed in 150 ml conical flasks. 1 cm x 8 cm filter paper strip (Whatman No. 42) was weighed along with the thread and dipped in the medium by hanging it with the thread. Each

flask was inoculated with 2 discs (3.0 mm diam. each) of the fungus from 8 days old colony. Flasks were incubated in duplicate at temperature 20°, 25°, 30°, 35° and 40°C for 8 days. After 8 days the filter paper strip was removed from the flask, cleaned gently with water and dried. Loss in weight of filter paper was calculated.

ii. Effect of pH on cellulolytic activity

30 ml of basal medium (without sucrose) was dispersed in 150 ml flasks. pH was adjusted in range of pH 3 to pH 8. 1 cm x 8 cm filter paper strip (Whatman No. 42) was weighed along with the thread and dipped in the medium by hanging it with the thread. The flasks were inoculated in duplicate with 2 discs (3.0 mm diam. each) of the fungus from 8 days old colony. Flasks were incubated for 8 days. After 8 days the filter paper strip was removed, cleaned gently with water, dried and weighed again. Loss in weight of filter paper was calculated.

iii. Effect of incubation period on cellulolytic activity

30 ml of sterile liquid medium was dispersed in 150 ml flasks. 1 cm x 8 cm filter paper strip (Whatman No. 42) along with thread was weighed and dipped in the medium by hanging it with the thread. 16 flasks were inoculated with 2 discs each (3.0 mm diam.) of the fungus

from 8 days old culture. Flasks were incubated at 30°C. Two flasks were opened every day from 8th to 15th day of incubation. The strips were removed, cleaned gently with water, dried and weighed again separately. Loss in weight of filter paper was calculated.

iv. Effect of Nitrogen sources on cellulolytic activity

30 ml of basal medium (with 0.5% CMC replacing sucrose) was dispersed in 150 ml conical flasks. Six different inorganic nitrogen sources ($NaNO_3$, KNO_3, NH_4NO_3, NH_4Cl, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$) were used. The quantity of nitrogen compounds replacing $NaNO_3$ in the Czapek-Dox basal medium was calculated so as to ensure the same amount of available nitrogen as that from $NaNO_3$. The flasks were inoculated in triplicate with 3 discs (3.0 mm diam. each) of the fungus from 8 days old colony. The flasks were incubated for 8 days. After 8 days the contents of the flasks were filtered. The culture filtrate was used to determine the cellulolytic activity in terms of reducing sugars produced by Dinitrosalicylic acid (DNS) method.

v. Effect of Nitrogen level on cellulolytic activity

30 ml of basal medium (with 0.5% CMC replacing sucrose) was dispersed in 150 ml conical flasks. $NaNO_3$ of the basal medium was replaced by different concentrations

(0.15%, 0.20%, 0.25% and 0.30%) of NH_4NO_3. The flasks were inoculated in triplicate with 3 discs (3.0 mm diam. each) of the fungus from 8 days old colony. After 8 days incubation the contents of the flasks were filtered and the culture filtrate was used to determine the cellulolytic activity in terms of reducing sugars produced by DNS method.

11. CELLULOLYTIC ACTIVITY IN MIXED CULTURE

The following combinations were studied in mixed culture:

i. Combinations of 2 isolates (10 combinations)
ii. Combinations of 3 isolates (9 combinations)
iii. Combinations of 4 isolates (5 combinations)
iv. Combinations of 5 isolates (one)

30 ml of Czapek-Dox basal medium with CMC (0.5%) as carbon source was dispersed in 150 ml conical flasks. Two discs (3.0 mm diam. each) from 8 days old culture of each isolate were inoculated as per above combinations. The flasks were incubated for 8 days. The cellulolytic activity was determined in terms of reducing sugars produced.