CHAPTER V

DISCUSSION AND CONCLUSIONS

The increasing demand of various types of paper has inevitably resulted in an increase in the number of paper and pulp industries in India in recent years. The production capacity of these mills has also been increased to meet the paper demand. The effluent from these industries passes through various stages like collection, sedementation, filtration, chemical treatment etc., before it is finally disposed. This effluent consists mainly of fibres (not retained in the filters), dyes, bleaching agents and other chemicals.

The present study was undertaken to obtain a general idea of fungal flora from the waters of some paper and pulp mills. The study bears a special reference to cellulolytic fungi because the effluent from these mills is rich in cellulose fibres. The cellulolytic activity of these fungi was studied in detail.

Three paper mills were selected for collection of samples. One of them (Nath Paper Mill) is located in Paithan (Aurangabad) which lies in Central Maharashtra. The other two factories are located in suburbs of Pune, of them one is the oldest factory in Maharashtra (Deccan Paper Mills Co., Ltd., established in 1887), and the other one is one of the biggest paper mills in Maharashtra (Pudumjee Paper & Pulp Co. Ltd.).

The portals of entry of fungi in the pulp and substrates in the mills are mainly air, raw materials and water used in the various processes. The air borne spores mix with water, settle on available suitable substratum and grow when favourable conditions appear.

Microscopic observation of small drops of samples revealed fungal spores and mycelial fragments, indicating thereby that fungi were in an active growing state. Jones (1974) suggested that mere detection of fungi in a sample was inadequate. It is essential to note the state in which they occur. The presence of fungal spores indicated passive occurrence of fungi, but the presence of mycelium indicated the active role of fungi in degradation of substratum and utilization of degraded material for their growth. Active fungi of this type are important organisms in the decomposition process.

The fact that a number of species of fungi are able to survive under conditions of extreme pollution indicates a major adjustment in their requirements. These fungi aid the purification of water by utilizing some of the pollutants during their growth and sporulation. This event creates a potential source of inoculum for plant diseases. This fact is documented in literature on fungal plant pathology from polluted waters. Cooke (1967) has reported Fusarium oxysporum, an active degrading agent of motor oil, to be a plant pathogen.

However, in our study, no serious disease incidence was reported by farmers who used polluted waters in their agriculture.

Cooke (1956a, b, 1957, 1967, etc.) was a pioneer in the study of fungi from polluted waters. He classified these fungi into two types (Cooke 1963) -

1. Hydrofungi: These are the true aquatic forms, and need free water for the completion of their life cycle. These are obligate fungi.
2. Geofungi: These are facultative aquatic fungi. They can survive in aquatic habitat but do not need free water for the completion of their life cycle.

Webster and Descals (1979) likewise opine that 'Aquatic Hyphomycetes' are not truely aquatic. They can grow on leaf litter or other substrata. They labeled such fungi as 'Amphibians.'

The fungi isolated in this study belong to the 'facultative aquatics' type of Cooke or the 'amphibian' type described by Webster and Descals.

In this study, 85 fungi belonging to 39 genera were isolated from the collected samples, using different media and methods. Most fungi belonged to Deuteromycetes (29 genera & 70 species). In addition there were Phycomycetes (4 genera & 8 species), Ascomycetes (5 genera & 6 species) and one species

of the genus Sclerotium from Mycelia Sterilia group, of the Basidiomycetes only Sporotrichum pruinosum was isolated.

The maximum number of fungi were isolated from Nath Paper Mill. Altogether 40 species (21 genera) of water fungi and 68 species (23 genera) of soil fungi were isolated from this factory.

Bait technique involving the use of straw and filter paper yielded exclusively cellulolytic fungi in pure forms. These could have been easily overlooked in agar plates due to their small colony sizes and slow growing nature. Maximum number of fungi were isolated by serial dilution method. Czepak-Dox agar medium was found to be most suitable for isolation of fungi. The use of different media and different techniques for isolation of fungi is necessary to obtain maximum number of fungi in culture. Weak media suppressed the growth of fast growing saprobes and allowed the growth of slow growing forms by allowing sufficient space and time and sparing the nutrients for expression of growth. Addition of chemicals like Rose Bengal to the medium also served the same purpose. Antibacterial antibiotics, suppressed the growth of bacteria which helped the development of pure fungal colonies.

Rhizopus nigricans, A. niger, Fusarium oxysporum, Paecilomyces varioti and Trichoderma viride were isolated from all the samples collected. Cunninghamella echinata, Mucor

species, aspergilli, penicillia, and fusaria, Syncephalastrum racemosum, Alternaria alternata, Cladosporium oxysporum, Curvularia species, Sporotrichum pruinosum, Stachybotrys pulchra and different species of Phoma were found very frequently in all the samples.

The fungi isolated by various methods were studied for their cellulose degrading ability. Treated cellulose is much easily digested by the organism, hence these fungal forms were grown in the medium with cellulose powder treated with orthophosphoric acid. The depth of the digested zone was measured. Out of the 84 fungi studied 47 species showed a definite cellulolytic activity. From the results obtained the isolated fungi were distributed in 4 groups -

Group I: Maximum activity or excellent decomposers which included fungi showing 20 mm or more depth of clearing zone after 30 days.

Group II: Moderate activity or moderate decomposers which included fungi with 10 - 19 mm depth of clearing zone after 30 days.

Group III: Poor activity or poor decomposers which included fungi with 9 mm or less depth of clearing zone after 30 days.

Group IV: Non-cellulolytic species. These are probably the secondary invaders.

Based on these observations three fungi, A. niger, S. pruinosum and T. viride from Group I were selected for further studies on their nutritional requirements and their ability to produce cellulases under various sets of conditions. In addition to these 3 fungi one species each from Group II and III were also selected for similar studies (M. lutea var. macrospora from Group II and H. grisea from Group III).

To select an appropriate medium for further studies and to observe growth and sporulation of these fungi on various media, these fungi were grown on 10 different (4 semi-synthetic and 6 synthetic) media. Considering the growth and sporulation of all fungi on all media, PDA and Czapek-Dox medium were found to be the most favourable media. However for physiological studies a medium with known composition is necessary, hence Czapek-Dox synthetic medium was selected as the basal medium for further studies.

The temperature study indicated that all the fungi were mesophilic except T. viride. Killing of the inoculum occurred at 50°C. Optimum temperature in all cases was found to be around 30°C. T. viride showed maximum temperature tolerance range whereas S. pruinosum was found to be most temperature sensitive fungus. Temperature tolerance range was wider for vegetative growth than for sporulation in all the five fungi.

T. viride and S. pruinosum preferred acidic medium whereas A. niger grew and sporulated well through the entire range of pH. In case of H. grisea and M. lutea var. macrospora double pH optima was noticed. Except for S. pruinosum the other four fungi showed change in pH of the medium after incubation. In A. niger pH shifted to acidic range while in H. grisea and M. lutea var. macrospora the medium became alkaline. In T. viride the shift was towards neutral. The change in pH of the medium may be due to the metabolites diffused in the medium during the growth of the fungus.

Ability of the five selected fungi to utilize carbon was studied by growing them in 15 different carbon sources. It was observed that monosaccharides and disaccharides were more easily utilized than polysaccharides. The carbon sources which favoured vegetative growth in these fungi induced sporulation to the same degree.

The selected fungi were grown in purely cellulose medium and their ability to produce cellulase under various environmental conditions was studied by changing temperature, pH and incubation period and by providing different carbon and nitrogen sources.

Four different methods were used to determine the cellulolytic activity of the fungi. In the viscometric method, loss in viscosity of CMC solution after addition of crude

enzyme was calculated. The reduction in viscosity was found to be directly proportional to the amount of enzyme produced.

The amount of glucose molecules produced during the incubation period was determined by estimation of reducing sugars (RS) (DNS reagent method) in the culture filtrate. Indirectly it reflects the cellulase enzyme activity because the amount of glucose molecules formed are directly proportional to the amount of enzyme released.

In the cup-plate method and Depth of Clearing zone method the digested zone in a cellulose medium is determined after inoculating the medium with crude enzyme and the fungus itself, respectively. The area of the digested zone is directly proportional to the activity of the fungus.

When filter paper is provided as sole source of carbon the enzyme released in C_1 and when CMC is added C_x is produced to break down the CMC molecules. Among the above methods Depth of Clearing Zone Method was followed by using pure cellulose powder and hence it gave the C_1 activity of the fungus. In the other 3 methods crude enzyme was used. C_1 or C_x activities were measured by changing the carbon source in the medium.

From the results obtained by the above four methods it was observed that enzymes of A. niger and M. lutea var. macrospora followed random cleavage of the cellulose molecule which

resulted in moderate loss in viscosity but not much RS was produced. In H. grisea exactly opposite behaviour was observed in which the enzyme released by the fungus directly formed RS by end splitting of the linear cellulose chains. This resulted in formation of comparatively more RS but it was not accompanied by loss in viscosity of CMC to the same degree.

T. viride and S. pruinosum showed that reduction in viscosity was accompanied by formation of appreciable amount of RS which indicated that the enzyme of these fungi may follow both modes of action i.e. random splitting and/or end splitting of cellulose molecule chain.

The effect of temperature on the enzyme activity of the selected fungi was studied in the temperature range of 20° to 40°C. All the fungi showed maximum activity in the range of 25° to 35°C and hence may be included in the mesophilic group of organisms having optimum temperature between 25° to 35°C except for T. viride which showed fairly good activity even at 40°C.

The activity of cellulases was studied in the range of pH 3 to 8. All the fungi under study showed varied pH optima but all of them preferred slightly acidic medium for their cellulase producing activity.

The fungi were incubated for 7 to 15 days to observe the effect of incubation period on cellulose degrading ability of fungi. In general all the fungi showed increase in cellulolytic activity with increase in incubation period.

The fungi were grown in 15 different carbon sources and it was observed that cellulases were produced only when CMC, cellulose powder or filter paper were provided.

When the cellulolytic activity of these fungi was studied in 6 different inorganic nitrogen sources maximum activity was observed in the presence of NH_4NO_3 except for M. lutea var. macrospora which preferred NH_4Cl.

Effect of nitrogen level on the cellulose degrading ability was also studied by varying the concentration of NH_4NO_3. A. niger showed increase in activity along with the increase in concentration whereas in M. lutea var. macrospora, S. pruinosum and T. viride the activity retarded with the increase in concentration. In H. grisea the activity increased with increase in the concentration upto 0.25% and thereafter it suddenly decreased.

The mixed culture study of these fungi showed that cellulolytic activity of the individual fungus was reduced. The vegetative growth as well as sporulation of the component fungi was found to be reduced.

85 fungi isolated from the samples collected are described at the end.

The present study yielded 3 taxa new to science (2 new species and one new variety), 5 species new to India and 18 new to Maharashtra; these are as follows:

New species:

1. Auriobasidium indicum sp. nov.
2. Dendrostilbella indica sp. nov.

New Variety:

1. Myceliophthora lutea Cost. var. macrospora var. nov.

New to India:

1. Mucor subtillissimus Oude.
2. Pseudeurotium multisporum (Saito et Minoura) Stolk
3. Alternaria dianthicola Neergard
4. Paecilomyces crustaceous Apinis & Chesters
5. Phoma leveillei Boerema & Bollen.

New to Maharashtra:

1. Cunninghamella elegans Lender
2. Chaetomium indicum Corda
3. Cladosporium macrocarpum Preuss.
4. Cladosporium sphaerospermum Penz.
5. Curvularia clavata Jain
6. Drechslera australiensis (Bungnicourt) Subram. & Jain ex Ellis, Subr. & Jain
7. Fusarium decemcellulare Birk.
8. Fusarium merismoides Corda

9. Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr.
10. Penicillium implicatum Biourge
11. Scolecobasidium variabile Barron & Busch
12. Sporotrichum pruinosum Gilman & Abbott
13. Stachybotrys pulchra Speg.
14. Trichurus spiralis Hasselbring
15. Coniothyrium fuckelii Sacc.
16. Pestalotia mangifolia Guba.
17. Phoma pomorum Thüm.
18. Phoma terrestre Saksena, Nand & Sarabhoy

Thus it may be concluded that there are many fungi occurring naturally in conditions ecologically modified by effluents. They degrade the surrounding substrata i.e. the substances which are considered as wastes from the industry. These primary invaders change the substratum conditions by their action making way to the secondary invaders. Other saprobes also make their appearance & further decompose the waste materials. The entire procedure however is slow.

The present studies indicated possibilities of employing such organisms under controlled conditions to degrade the cellulosic wastes. However the biological solution of any problem is not so simple. The filamentous fungi produce large amount of mycelium and dry spores mixing with the substratum and air respectively. Hence, the release of such fungi in nature for inducing biological degradation of

wastes is always premature until the intensity of possible side effects or harmful aspects of fungal growth are well studied. Such a step is likely to turn into a new hazard than a solution to the original problem.

The results obtained in the present studies are suggestive that certain organisms like A. niger, H. grisea, M. lutea var. macrospora, S. pruinosum and T. viride when introduced singly or in combination may prove to be beneficial in the fibre or cellulose degradation process, which is a problem for factory organisers and for the agriculturists who use these wastes in their fields. The fibres may get accumulated on the soil and form a thick layer which hinders the growth of the plants. The cellulase activity was found to be amenable of considerable alteration by changing physical and nutritional conditions, as evident from the data presented in the foregoing pages.

It is not possible at this stage to reccommend any particular species or group of species for practical use, as some more data based on adequate experimental evidence is mandatory. This study, though preliminary in its investigation, definitely opens up a new arena for further work on microbial degradation of cellulosic wastes by filamentous fungi.