The isolation of fungi from barley straw under alkaline conditions and their utilization as agents of biodegradation

by

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A thesis submitted to the University of Aston in Birmingham for the degree of Master of Philosophy

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SUMMARY

By employing selective techniques, cellulolytic, xylanolytic and ligninolytic fungi have been isolated from barley straw under alkaline pH conditions and at a range of incubation temperatures. The most common isolates were identified as Alternaria tenuis, Aspergillus fumigatus, Chaetomium globosum, Cephalosporium sp. Coprinus cinereus, Fusarium sp., Scopulariopsis brevicaulis and Streptomyces sp. The fungi Aspergillus fumigatus and Cephalosporium sp. have been isolated at all the temperatures used (20°C., 25°C., 30°C., 35°C., 40°C., 45°C and 50°C); the rest of the fungi have been reported up to 35°C.

Fourteen different vegetation samples were collected from various regions of Birmingham, in the summer 1976. Subsequently the isolation of Coprini from these samples showed the presence of C. cinereus and C. lagopus while two isolates of Coprini were not identified to species levels.

Isolated fungi were assessed for the utilization of different carbon sources, i.e. cellulose, xylan, ball-milled straw, phenolic acids including gallic acid and tannic acid, and lignins (lignosulphonate, Indulin A-R, and the lignin prepared by 72% H_2SO_4 method). Some of the fungi such as A. fumigatus, C. globosum, C. cinereus, and Botryotrichum piluliferum have shown extensive growth and very strong activities on the media supplemented individually with either of the above mentioned carbonaceous compounds as the sole sources.

The fungi which showed good utilization of different carbon sources were tested for the colonisation on the barley straw under alkaline pH conditions and at a range of incubation temperatures using aseptic and septic techniques. The most rapid substrate colonisation under these circumstances were shown by C. cinereus or by B. piluliferum. Nevertheless, their substrates colonisation rates were gradually decreased above 35°C.

Synergistic and antagonistic actions of some of the rapid substrate colonisers were studied. C. cinereus or B. piluliferum were found to be the most dominant colonisers on straw and responsible for the suppression of other fungi when either of these fungi was inoculated with them under sterile conditions. However, when non-sterile conditions prevailed, the rapid growth of C. cinereus or B. piluliferum on straw almost totally suppressed the appearance of most of the other fungi.

KEY WORDS

Isolation, Colonisation, Cellulolytic, Ligninolytic, Fungi

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DECLARATION

This work was carried out between I975- I978

at the University Of Aston in Birmingham.

It has been done independently and has not been

submitted for any other degree.

Syed Kazim Quli

SYED KAZIM QULI.

Dedicated to my late grandfather

Syed Ajaz Husain Jafri, B.A. (Hons.)., Ph.D (London).

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CHAPTER ONE

INTRODUCTION

Chapter 1

1. Introduction

1.1. Thesis Proposal

The object of this thesis is to investigate the aspects of biodegradation of barley straw under alkaline conditions by a range of selected fungi. It is hoped that the result of a biodegradation process will be a product with an increased digestibility and palatability to ruminants by virtue of selective decomposition of certain components of the lignin carbohydrate complex present in the straw.

The selection of fungi will be made after isolating a number of potentially useful biodegrading fungi from barley straw under the influence of alkaline pH and at a range of temperatures. The reasons for using alkaline growth conditions are twofold. Firstly, it enables the isolation of those cellulolytic fungi which can tolerate alkaline pH conditions in their growth environments; and, secondly, it enables the up-grading of straw under non-sterile conditions using selected fungi.

The scheme of this research investigation is outlined as follows:

1. The isolation of fungi from barley straw under alkaline conditions.
2. Isolation of <u>Coprinus</u> species from a range of vegetations.
3. Influence of a range of temperatures on the isolation of fungi under alkaline conditions.
4. Utilization of various carbon sources by the isolated fungi.

5. Colonisation of barley straw by selected fungi under sterile and non-sterile conditions.

1.2. Straw - A potentially valuable cereal by-product

The increase in cereal production has resulted in a surplus of straw for which farmers have no economical outlet. In Great Britain, most straw is disposed of by ploughing-in or burning; however, burning causes pollution problems such as smoke and air-borne ash. These methods of disposal are undesirable because of the waste of valuable carbohydrate components, shielded from ruminant digestive system by the high lignin content of mature straw. The lignin carbohydrate complex represents 50% of the primary production of the plant, and it is thought that this potentially valuable product could be up-graded using micro-organisms for the production of a product suitable as a ruminant feedstuff (Seal, Eggins, 1972; Phoenix, Bilanski, W.K., Mowat, 1974; O'Callaghan, 1975; Peitersen, 1975).

1.2.1. The Chemical composition of straw

Rege (1927) identified the consituents as cellulose, hemicellulose, miscellaneous pentosans, structural proteins, lignin and other minor components. However, the recent work carried out by Peitersen (1975) on the analysis of barley straw showed the presence of 75% carbohydrates, which consisted of cellulose (40%) pentosans (31%); lignin (17%) and 0.5% nitrogen, etc.

1.2.2. The use and disposal of straw

As mentioned earlier, there has been a substantial increase in cereal production due to the increase in world population. Since the turn of the century, there has been a large increase of land under arable crops in England and

Wales, and the gradual increase in the production of barley, wheat and oat crops has caused a surplus production of straw. According to the report of the National Farmers Union (NFU, 1973) Working Party, on the use and disposal of straw, 9.3 million (m) tons per annum straw production was estimated in the arable area of England and Wales. Of the total production, 3.55 m tons or 38.2 per cent was surplus to the requirements. Figures provided by the Advisory Council for Agriculture and Horticulture (A.C.A.H., 1973) in England and Wales on Report on straw disposal showed the following estimations:

Feeding	14 m tons (15.0%)
Bedding and crop storage	3.40 m tons (36.4%)
Interfarm sales	0.86 m tons (9.3%)
Burned	3.42 m tons (36.6%)
Ploughed-in	0.15 m tons (1.6%)
Non-agricultural use	0.10 m tons (1.1%)

Excessive straw is a greater problem in the eastern counties due to an increase in intensive cereal production at the expense of livestock production, whereas in the western counties, livestock production remains the most important farming activity. It can be seen that the excess amounts produced in the eastern counties would be of some value to livestock producers in the west if the transportation of this bulky low-cost commodity could be made economical.

To overcome this disposal problem, a number of organisations and working parties have been established. The functions of the formed organisations were based upon the supply of technical evidence and expertise (NFU, 1973; A.C.A.H., 1973)

1.2.2.1. Agricultural and non-agricultural usage

Straw has limited uses in agriculture and industry, some

of them are as follows:

a) use as animal bedding

b) as an insulator for crop storage

c) as a material for making straw board

d) as a packing material

e) as a pulp additive

f) as a brick filler.

1.2.3. Up-grading of straw

Lignin is found in mature straws and its presence causes the decrease in acceptability or digestibility of straw in the rumen of ruminants. The rumen flora is capable of breaking down cellulose, but is unable to degrade lignin (Van Soest and Merleus, 1974). However, investigations have been made by many workers to find a viable method to improve the feeding value of straw by increasing its digestibility to ruminants. The chopping or grinding of straw increases its palatability and digestibility to ruminants, but high capital and running costs for the equipment does not encourage the operation of such mechanical processes. However, the chemical treatment of straw using NaOH (the Backman process) is known to increase the availability of the carbohydtrate fraction of straw to ruminants. Although chemical treatment is a valuable process for improving the feeding value of straw, the technique possesses many drawbacks and safety hazards, as sodium-hydroxide is very caustic (Chandra and Jackson, 1971; Carmona and Greenhalgh, 1972 and Palmer, 1976).

Both physical and chemical methods as outlined above have shown the energy consumption and the practical handling problems. This has suggested that a more feasible method is still required to improve the feeding value of straw.

1.2.3.1. Biological up-grading

The main criteria for the operation of straw up-grading process is that it should be economical, safe and the up-graded end-product should be acceptable and of enhanced food value to ruminants.

As mentioned earlier, a large proportion of straw is unavailable to ruminants, because of the highly crystalline nature of the cellulose molecule and also the existence of a lignin-carbohydrate complex (Bjorkman, 1957). However, if convenient methods can be found to enhance the availability of this material, a large quantity of straw could be supplemented in the diet of ruminants. This could also solve a considerable amount of disposal problems.

The purpose of biological up-grading of straw is to increase the digestibility of straw to ruminants. A small reduction in lignin content would probably provide a greater availability of cellulose content to ruminants and thus could also help to increase the digestibility in the rumen (Itan, Dunlop and Callihan, 1971; Rogers, Coleman, Spino and Purcell, 1972; Eriksson, 1974; and Baker and Millett, 1975).

1.3. Fungi as the agent for up-grading processes

The preference for fungi over bacteria as the agents for an up-grading process is due to the fact that the fungi rapidly colonise large surface areas of carbohydrate-based materials, and they require low moisture conditions for semi-solid fermentation. There are many cellulolytic fungi capable of using the large amounts of cellulose in agricultural waste as an energy source. They occur naturally in animal and

plant wastes and are obligate aerobes and do not produce the unpleasant odours usually associated with anaerobiocity. pH optima for such fungi tend to discourage bacteria which are pathogenic. Moreover, they are also good sources of protein, being low in nucleic acid levels. Their mycelial habitat gives them a colonizing advantage in that they can spread over solid substrates and penetrate intact tissue without any pre-treatment.

1.4. Role of temperature and pH upon the growth of fungi

Temperature and pH play key roles on the growth activities of fungi.

1.4.1. Effect of temperature

It is apparent from physiological studies of fungi that the role of temperature is very important as it affects metabolic activity, spore germination, mycelial growth and reproduction (Cochrane, 1958). With regard to their growth temperatures, fungi, as well as other micro-organisms, may be divided into three groups: phychsophilic, mesophilic and thermophilic. The first group include the species of fungi which can tolerate temperatures below $0^{\circ}C$. The second group are able to grow between $20-40^{\circ}C$ while the third group are capable of growing between $20-50^{\circ}C$ (Cooney and Emerson, 1964).

It is quite obvious from previous investigations that different species of fungi from the same group have different temperature requirements for their maximum or minimum growth activity. However, this temperature requirement for the same species of fungus could be variable in two different media due to the influence of other factors, i.e. pH and components of media and their moisture content, etc. (Humphrey and Sigger,

1933; Cartwright and Findlay, 1934).

1.4.2. Effect of pH

Hydrogen ion concentration (pH) has a profound affect on fungi growing on any natural or synthetic medium. Change in this parameter for growth could alter the ion availability, cell membrane permeability, enzyme action and subsequent growth of the fungus. It is noteworthy that different groups of fungi possess different ranges of pH for their growth optima. However, this could depend upon the type of media, incubation temperature and environmental conditions at which fungi grow (Wolpert, 1924; Humphry and Sigger, 1933; Weindling, 1938; Allsopp, 1973 and McShane, 1976).

1.5. Fungal colonisation on straws

Fungal colonisation on straw generally results in the rapid formation of a mycelial mat covering a large surface area believed to be the result of exogenous enzyme action. In other words, enzymes are released by organisms during substrate colonisation and diffuse into different constitutional regions of the material, whereby the process of decomposition occurs. The enzymes responsible for the decomposition processes are presumably cellulases, hemicellulases and polyphenoloxidases (or laccase).

Garrett (1951, 1963) studied the fungal colonisation on plant debris and divided the isolates on the basis of utilisation of different components in the material, i.e. simple sugars, cellulose, hemicellulose and lignin, etc. He used the term 'sugar fungi' for those primary colonizers which have failed to utilize the rest of the named components except the sugars, whereas he called secondary fungi to those which were able to attack all the components in plant debris. He

observed that most of the former colonisers were phycomycetes; whereas the latter were found to be ascomycetes and basidiomycetes (Burges, A., 1939).

Rege (1927) has studied the colonisation of fungi on sterile rice straw by inoculating a mixture of three different isolates, i.e. Aspergillus sp., Acremoniella sp and Coprinus sp. He observed during 6-20 days incubation period that, though the first two fungi were visible on inoculated material, the third, i.e. Coprinus sp. was dominant in all the incubated bottles. This was shown in the form of a solid mass and intertwined intimately with the material. Eastwood (1952) has also reported the dominant colonisation of a species of Coprinus lagopus, in the form of larger mycelial growth on composted barley straw. She had also noticed another fungal isolate: Chaetomium globosum which was also competing with the former organisms on the same substrate. Chang and Hudson (1967) have also reported the isolation of dominant colonisers, i.e. Coprinus cinereus, C. megacephalus, and Chaetomium thermophile, from the composting material of wheat straw. Although other fungi colonised these substrates and have been isolated by the named investigators, the stated fungi mostly dominated by covering a larger area of the substrate by their rapid mycelial growth. Along certain fungal species these workers have analysed the degraded straws to study the utilization of different components and have found a high percentage weight loss in the cellulosic content of straw. These investigators have studies the above-mentioned isolates in pure cellulose and also compared their cellulolytic activities with other groups of fungi isolated from the straw, but again have come to the same conclusion that the above outlined fungi were highly cellulolytic on both natural composting materials and on culture media.

Previous workers at the Biodeterioration Information Centre (B.I.C.), at the University of Aston in Birmingham, have reported the rapid colonisation by *Coprinus cinereus*, on alkali treated non-sterile barley straw. Pure culture studies have shown that this fungus is capable of growing under highly alkaline pH conditions and that it tolerates high incubation temperatures (Seal, 1973 and McShane, 1976).

Other workers have shown that *Chaetomium globosum* and *Botryotrichum piluliferum* are lignocellulolytic and grow over a wide range of temperatures and pH. Levi and Preston (1965) have reported 75% reduction in lignin content when *Chaetomium globosum* was inoculated into wood placed in mineral salt medium. Haider and Domsch (1969) have reported a good utilisationof cellulose on lignin by *Botryotrichum piluliferum*, when it was growing on straw. Decomposition of starch, pectin xylan and CMC was also reported by this strain by Borut (1960); Domsch (1960) and Jensen (1931).
Haider and Domsch (1969) have also reported a loss in weight and tensile strength of maple wood strips when *Botryotrichum piluliferum* was growing on it. It is interesting to note that this organism can tolerate 40°C (Blochwitz, 1914) and can still grow above pH 8.8 (Jensen, 1931).

1.5.1. Isolation of Fungi from Straw

Many techniques have been developed for the isolation of fungi onto culture medium. These techniques are mostly divided into general and selective methods. The isolation techniques described by several soil microbiologists are outlined below:

A. General techniques:

1. The soid dilution plate (Garrett, 1951)

2. The Warcup soil plate (Warcup, 1950, 1960)
3. Chesters soil immersion plate (Chester, 1940, 1948)
4. Thornton's screen immersion plate (Thornton, 1952)

B. Selective techniques:

5. Methods of Harley and Waid for root-surface fungi,or the washing technique (Harley and Waid, 1955).
6. Warcup's hyphal isolation method (Warcup 1956, 1960)
7. A screen substrate method (Eggins and Lloyd, 1968)
8. Perfusion technique (Malik and Eggins, 1970)

By applying the dilution plate method, Eastwood (1952) isolated a number of different groups of fungi from the composts of fresh lawn mowings and cut barley straw. She isolated a number of thermophilic bacteria, but failed to isolate any thermophilic fungi from the composts at higher temperatures. Thermophilic fungi were defined by Cooney and Emerson (1964) as 'one that has a maximum temperature for growth at or above 50°C'. According to Crisan (1959) 'Thermophilic fungi can be placed according to optimum temperature for growth which should lie at or above 40°C'.

Chang and Hudson (1967) have reported a number of mesophilic and thermophilic fungi from wheat straw compost using dilution and washing techniques for isolation. Some other investigators have also reported the isolation of different mesophilic and thermophilic fungi from straws. (Fergus, 1964; Flannigan, 1969 a and b; 1970; and Ogundana, 1975).

It is interesting to note that although many recommended techniques have been employed by many workers for the isolation of fungi from compost, most of these investigations have been seen working within the acid pH range, because it was assumed

that acid conditions were more conducive to fungal development. However, an attempt has been made in this investigation for conducting an isolation and colonisation programme of fungi under alkaline conditions with the reasons previously stated. Emphasis was also made on the role of temperature during isolation and colonisation of fungi on straw.

1.6. Programme of work

The work in progress at the B.I.C. to develop a feasible technique for the up-grading of ligno-cellulose waste materials. The purpose of this work is to up-grade the material by converting it into a possible feedstuff to supplement the diet of ruminants. The end-product, should, however, be palatable and acceptable, have an improved digestibility and nutritional value, and an increase in protein content.

The work presented in this thesis is a part of the investigation being carried out at the B.I.C. for the biological up-grading of straw. This work is concerned with the isolation of fungi from barley straw under alkaline conditions and their utilisation as the agents of biodegradation (as mentioned earlier). For this study a number of different groups of fungi have been isolated from barley straw at a range of incubation temperatures. Their lignocellulolytic activities have been assessed, and finally fungi have been selected as agents for biological up-grading processes.

CHAPTER 2

Isolation of fungi from barley straw under alkaline conditions.

CHAPTER 2

2.1. Introduction

This part of the study was concerned with the isolation of fungi from barley straw after providing alkaline conditions for their growth. The purpose of achieving this technique was to facilitate growth conditions only for those fungi which could tolerate high pH's in their immediate growth environment. This would also provide appropriate criteria for selecting some specific isolates to use as the agents for the up-grading of waste or surplus amounts of straw.

During the biodegradation process, there would be less chances of competition from other fungi, which do not possess the ability to grow under the defined conditions (i.e. under high pH's).

Selective media were chosen for the isolation of fungi which were able to grow under the conditions outlined above. Emphasis was laid on the isolation of alkalophilic organisms which could also utilize lignocellulosic material. This will be discussed in more detail in the relevant chapter.

2.1.1. Isolation of fungi from straw under alkaline conditions

Throughout the fungal isolation programme, no other varieties of straw were used except the straw from a Hassan variety of spring barley harvested in 1975 from sandy loam soil of Harleston, Northamptonshire, England.

A number of techniques have been employed by previous investigators for the isolation of fungi from various types of straw. Some of the commonest were direct inoculation, (Waksman, 1916), the dilution plate method (Waksman, 1927;

Brierly, Jensen and Brierley, 1927; Warcup, 1950, 1951, 1955 and 1960) the Harley and Waid (1955) washing method for root-surface fungi, and the hyphal isolation method of Warcup (1955).

The direct inoculation method was originally described by Waksman (1916) to demonstrate whether fungi produced mycelium in the soil or were deposited there from the air as spores and remained viable but inert. On conclusion of his experiment, Waksman found that the mycelial development from the lumps of solid was too rapid to have been from germinating spores, and must have arisen from pre-existing mycelium within the soil.

The dilution plate method was developed by earlier soil microbiologists form the isolation of different species of micro-organism in pure culture (Waksman, 1927). This method consisted of shaking up a knownquantity of soil crumbs in sterile water, either by hand or by a mechanical shaker and finally mixing the diluted suspension of material with the sterile shake medium.

Washing techniques were employed by most workers for the isolation of slow growing fungi from plant material which were able to colonize, but were unable to develop active mecelial growth when the material was directly suspended onto agar plates, since fast growing fungi suppressed their growth by covering the entire agar surface.

To overcome these problems, Harley and Waid (1955), have described a washing technique for studying active mycelial fungi present on living roots and other surfaces in the soil. They carried out several washings of plant material placed in screw-topped phials containing a standard amount of

sterile distilled water. The phials were agitated on a mechanical shaker for two minutes, with an excursion of 4.0 cm. After the completion of each washing period, water was decanted from the phials by replacing with fresh aliquots and the phials were shaken again. Some batches of plant materials were washed for 20-30 times by repeating the whole process.

The reason for repeated washing was to ensure the detachment of fungal spores and other propagules from the substrate. These were examined by inoculating the washings on agar plates by the dilution plate method (Warcup, 1955a, 1957). To find the difference in fungal isolation from the washed and unwashed materials, the inoculation was made by plating out both former and latter materials on different selective growth media.

In the present study a direct inoculation and washing technique has been employed for fungal isolation. The pH of the media was maintained entirely under alkaline ranges and this was achieved by the use of ammonia and sodium hydroxide (NaOH) solutions. This showed that there was less chance of isolation and growth of acidophilic fungi by the application of alkaline pH conditions. In this part of the investigation an attempt was made only for the isolation of mesophilic fungi.

2.2. EXPERIMENTAL PROCEDURE

Random samples of straw were collected from both the inner and outer surfaces of a bale of straw stored in the shed of the Biodeterioration Information Centre (B.I.C.) premises. The materials were cut into equal sized pieces, i.e. approximately 3-4 cm long. The entire operation was carried out under aseptic conditions to control contamination.

2.2.1. Fungal isolation method

The following methods were employed for the isolation of fungi from straw:

1) Direct inoculation method
2) Inoculation of washed straw
3) Inoculation of washings from straw
4) Inoculation of ammonia treated straw

2.2.1.1. Direct inoculation method

In this part of the fungal isolation, the inoculation of straw was made onto two types of agar media:

i) the inoculation onto different aqueous ammonia (v/v) concentrated mineral salts agars; and
ii) the inoculation onto cornmeal agar, malt agar, glucose-cellulose agar, glucose-starch agar and potato dextrose agar.

i) The first type of agar media were prepared by aseptic addition of 1, 0.5, 0.25, 0.125 and 0.0625% aqueous ammonia by volume into previously autoclaved individual sets of mineral salt agar media. Each set of medium was prepared without any addition of a carbon and nitrogen component. The reason for not adding any carbon source was to facilitate the

appearance and the growth of fungi which would utilize straw components as the only carbon source when straw pieces were inoculated onto agars for fungal isolation. The purpose of adding a range of aqueous ammonia was twofold: 1) to obtain a range of alkaline pH media without the addition of a buffering system (see Table 1 for pH obtained) and 2) to examine the isolation of fungi under the influence of aqueous ammonia supplemented in the growth media. However, prior to ammonia supplementation, each set of medium contained 1 gramme (g) by weight KH_2PO_4: 0.5g. K_3PO_4: 0.2g. $MgSO_4$: 0.1g $CaCl_2$ and 15 gms Oxoid agar added to 1 litre distilled water. 30 mg (w/v) Rose bengal was also added in each set of medium to suppress the bacterial growth (Ottow, 1972) during fungal isolation from inoculated straw pieces onto agar plates. After preparation of agar media 20 ml aliquots from each agar were poured separately into sterile petri dishes. After agar had set on plates, five pieces of straw were placed onto the surface of each agar. This was repeated for all dishes containing different concentrations of ammonia solution. The inoculated plates were incubated for an appropriate period of time for the isolation of mesophilic fungi. However, six replicates were used for each ammonia concentrated agar medium to facilitate maximum probability for the isolation of different groups of fungi under these circumstances.

ii) In the second type of media, cornmeal agar, malt agar and potato dextrose agar were prepared according to the directions provided by the commercial manufacturers(see appendix for media preparation). While the components added for preparation of glucose-cellulose agar and glucose-starch agar were the same as used by Eggins and

Pugh (1962) for their cellulose agar, except the addition of glucose or starch along with cellulose.

a) <u>Glucose-cellulose agar</u>

Potassium dehydrogen orthophosphate	1.0 g
Ammonium sulphate	0.5 g
Potassium chloride	0.5 g
Yeast extract	0.5 g
L. asparagine	0.5 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Oxoid agar	15. g
Ball-milled cellulose suspension (4.0%)	125 ml
Glucose	5 gm
Distilled water	to 1 litre

b) For the glucose-starch agar the cellulose suspension was substituted with 5g glucose and 5g starch.

The glucose was sterilized by using millipore filtration after dissolving it in a measured amount of distilled water. This was to avoid caramelization of glucose during the autoclaving process. The sterile glucose solution was incorporated aseptically with its respective agar medium previously autoclaved. However, the rest of the agar media were autoclaved as usual and the pH of these agars were adjusted to alkaline range by aseptic addition of 1N sodium hydroxide. (See Table 1 for the pH obtained.) The media were cooled to 45°C and 20 ml aliquots were poured into sterile petri dishes and allowed to set.

Table 1

pHs of different media

Agar and liquid media prepared	pH adjusted to:
Mineral salt agar with 1% NH3	10.10, 10.30*
Mineral salt agar with 0.5% NH3	9.90, 9.80*
Mineral salt agar with 0.25% NH3	9.45, 9.00*
Mineral salt agar with 0.125% NH3	8.70, 8.60 *
Mineral salt ager with .0625% NH3	7.85, 7.88 *
Corn meal agar	7.70, 7.00
Cellulose agar	7.70
Cellulose/glucose agar	7.70, 7.00
Glucose/starch agar	7.00, 8.60
Malt agar	7.70, 9.00
Potato dextrose agar	7.70, 9.00

* indicates liquid media

A similar method was employed as has been outlined in section 1) for fungal inoculation and incubation of these media.

After a certain period of incubation, the isolated fungi from both types of inoculated agar media were sub-cultured onto cellulose agar (Eggins and Pugh, 1962) for pure culture studies and for further identification (Gilman, 1957; Barron, 1958; Nobles, 1964; Barnett, 1972). The reason for using cellulose agar was, to encourage the growth of cellulolytic fungi. Cellulolytic activities of isolates will be discussed in Chapter 5.

Examination of isolates was carried out as follows: by direct viewing of the agar; by looking through the magnifying hand lens,and under the low power dissecting microscope. Also by viewing the detached fragments from suspended straw pieces after placing on clean glass slides under low and high magnification light microscope, or by making the proper slides of the organisms by using colour stains, e.g. lactophenol cotton blue.

2.2.1.2. pH measurement of inoculated agars

pH estimation was carried out during fungal isolation on agar media. This was achieved by scraping off the fungal growth from the agar surface, small agar blocks were collected and were placed in washed and oven dried screw topped universal bottles containing a few neutral glass beads(which help in breaking up the agar blocks).

Equal amounts of distilled water were also incorporated into the universal bottles. The contents were shaken either by hand or by using a mechanical shaker to obtain homogeneous suspension of agar and water. The pH measurement of material was carried out on a Pye temperature compensated pH meter with glass electrode,and an average reading from five replicates for each agar medium was considered as standard pH value.

2.2.2. Inoculation of washed straw

A similar procedure was employed for the collection and cutting of straw as mentioned in Section 2.2. Ten straw pieces were placed aseptically into screw topped universal bottles containing approximately 10 ml of sterile distilled

water. After that, washing techniques (Harley and Waid, 1955) were carried out by fixing screw capped universal bottles to the clamps of a flask shaker. The machine was turned up to an appropriate speed for at least a half minute for the first washing, 1 minute for the second, 1½ minutes for the third, 2 minutes for the fourth, 2½ minutes for the fifth and finally 3 minutes for the sixth washing.

After completion of the first washing, the materials were transferred aseptically into another container having the same amount of distilled water as was used in the first washing. The same procedure was applied for the second washing and this was repeated continuously up to the completion of six successive serial washings. After completion of the final washing, the washed straw pieces were placed on to different ammonia concentrated mineral salt agars. The rest of the techniques employed were the same as have been described for the direct inoculation method. (See 2.2.1.1.)

2.2.3. Inoculation of washings from straw

This inoculation method was adopted to isolate the fungal propagules which were present as surface contaminants on straw at the time of sample collection and can be easily detached from the material after applying a serial washing technique and finally deposited into washing water.

All six serial washings collected aseptically from the previous method were inoculated on glucose/cellulose agar, glucose/starch agar and potato dextrose agar, each incorporated with 30 mg rose bengal/litre. However, a one ml aliquot was taken from each washing sample by using sterile 5 ml syringes and the material was spread on the

surfaçe of agar plates. To make sure that the content was spread properly on the entire surface, each plate was rotated gently on the bench of inoculation cabinet. To provide maximum chances of isolation, five replacates were prepared by plating out each dilution on each agar medium, and the inoculated plates were incubated at 30°C for a certain period.

2.2.4. Inoculation of ammonia treated straw

The purpose of this part of the work was to examine and isolate those indigenous or non indigenous fungi which were present on the straw at the time of sample collection and were able to grow under the influence of different ammonia concentrations.

Aseptically collected random straw samples were placed in a selected number of sterile 1 lb capacity jam jars with approximately two grammes of material per container. After that, five different aqueous ammonia concentrated mineral salt solutions were added into five different sets of jars.

Five replicates were prepared for each ammonia solution, and the amount of solution incorporated into every jar was sufficient to soak the contents properly and also to provide appropriate conditions for a semi-solid fermentation process. Having done the entire process under aseptic conditions, the jars were incubated for the isolation of mesophilic fungi after covering their tops with sterile aluminium foil.

The ammonia treated incubated straw peices were viewed microscopically for the appearance of fungi. After having done that, a direct inoculation of straw pieces was made by placing them on the surface of cellulose agar plates. Afterwards, a similar procedure was employed for subculture and

pure culture studies of fungi as was used in section 2.2.1.1.

2.3. RESULTS

2.3.1. Fungi isolated from direct inoculation methods

1) Plating out on different ammonia supplemented mineral salt agars

Tables 2 and 2a summarise the results for the isolation of fungi and the final pH measurement of growth media. The most common micro-organisms isolated in this technique were Aspergillus fumigatus, Chaetomium globosum, Scopulariopsis brevicaulis and Streptomyces sp. Their appearance seemed to be almost constant in all the ammonia concentrated agars. However, no attempt was made to examine their frequencies of occurrance on agar plates. There were also occasional appearances of Alternaria tenuis, Cephalosporium sp. and Fusarium sp. These fungi were predominant inhabitants on straw as reported by previous investigators (Walker, 1941; Webster, 1956, 1957 and Webster, 1958). However, there is good reason to believe that the presence of ammonia in agar could probably have an inhibitory effect on their growth, but no attempt was made to investigate this.

The pH's of all inoculated agars were still shown under alkaline ranges, which suggests that the isolated fungi were capable of growth under highly alkaline pH's. It was one of the prime aims to isolate high pH tolerating fungi in this study.

11) Plating out on glucose/cellulose, glucose/starch on Corn meal agar, on malt agar and on potato dextrose agar

See the summarised results in Tables 3 and 3a.

Table 2. Fungi isolated from straw pieces directly inoculated onto mineral salt agars supplemented with different concentrations of aqueous ammonia at 30°C.

FUNGI	Fungal growth on agars supplemented with different percentages of aqueous ammonia									
	1%		0.5%		0.25%		0.125%		0.0625%	
	1st weeks	2nd weeks	1st weeks	2nd weeks	1st weeks	2nd weeks	1st weeks	2nd weeks	1st weeks	2nd weeks
Alternaria tenuis	-	-	-	-	+	-	-	+	-	-
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+
Aspergillus spp	-	-	-	+		+	+	+	+	+
Chaetomium globosum	+	+	+	+	+	+	-	+	+	+
Chaetomium sp.	-	-	-	+	-	-	-	-	-	-
Cephalosporium sp.	-	-	-	-	+	-	-	+	-	-
Coprinus cinereus	-	-	+	+	+	+	+	+	+	+
Fusarium sp.	-	-	-	-	-	-	+	-	+	-
Penicillium sp.	-	-	-	-	-	-	-	-	-	+
Scopulariopsis brevicaulis	-	+	+	+	+	+	-	-	-	-
Streptomyces sp.	-	+	+	+	-	-	-	+	-	-
Rhizopus sp.	-	-	-	-	-	-	-	-	+	-
Scopulariopsis chartarum	-	-	-	-	-	-	+	+	-	-
Scopulariopsis sp.	-	-	-	-	-	-	+	-	-	-

- = Negative growth
+ = Positive growth

Table : 2a

pH measurement of mineral salt agars supplemented with different concentrations of aqueous ammonia, after the growth of isolated fungi which appeared from the direct inoculation of straw pieces after two weeks incubation at 3o^{o}C.

Percentage of aqueous ammonia in agar media	pH of agar media		
	Initial pH before inoculation	After one week inoculation	After two week inoculation
I % NH_3	I0.30	8.80	8.85
0.5 % NH_3	9.90	8.65	8.45
0.25 % NH_3	9.45	7.45	8.20
0.I25 % NH_3	8.70	8.I5	8.00
0.0625 % NH_3	7.85	8.I0	8.20

Table 3. Fungi isolated from straw after direct inoculation onto different agar media, after incubation at 30°C, for 10 days

FUNGI	Appearance of isolated fungi on different agar media									
	On corn-meal agar with pH:		On glu-cose starch agar with pH:		On malt agar with pH:		On pot-ato dextrose agar, pH:		On gluc-ose-cell-ulose agar with pH:	
	7.70	9.00	7.70	9.00	7.70	8.60	7.70	9.00	7.70	9.00
Alternaria tenuis	+	+	+	+	+	+	+	+	+	+
Alternaria sp.	-	-	+	-	-	-	-	-	-	-
Aspergillus fumigatus	-	+	+	-	+	-	+	+	+	+
A.versicolor	-	-	-	-	-	-	-	-	+	-
Aspergillus spp.	+	-	-	+	+	-	-	-	+	-
Arthrobotrys sp.	-	-	-	-	-	-	+	-	-	-
Aureobasidium pullulans	+	+	-	+	+	-	+	+	-	+
Botrytis cineria	-	-	+	+	+	+	+	-	-	+
Botrytis sp.	-	-	-	-	-	-	-	+	-	+
Cephalosporium sp.	+	-	+	-	+	+	-	-	+	-
Chaetomium globosum	+	+	+	+	+	+	+	-	+	+
Chaetomium sp.	-	-	+	-	-	+	+	+	-	-
Cladosporium sp.	-	-	-	-	-	-	+	-	-	-
Coprinus cinereus	-	+	-	+	+	+	+	+	+	+
Cunninghamella ellegans	-	-	-	-	-	-	-	-	+	-
Fusarium spp.	-	+	-	-	+	+	-	+	+	-
Humicola sp.	-	-	-	-	+	+	-	-	-	-
Mucor sp.	-	-	-	+	-	-	+	+	+	+

Table 3 (Cont)

FUNGI	Appearance of isolated fungi on different agar media									
	On corn-meal agar with pH		On glu-cose-starch agar with pH		On malt-agar with pH		On pot-ato-dextrose agar with pH		On glu-cose-cellulose agar with pH	
	7.7	9.0	7.7	9.0	7.7	9.0	7.7	9.0	7.7	9.0
Paecilomyces sp.	-	-	-	+	-	-	+	+	-	-
Penicillium sp	+	+	+	-	-	+	+	+	-	+
Rhizopus sp.	-	-	-	-	-	-	+	-	-	-
Scopulariopsis brevicaulis	+	-	+	-	+	-	+	+	-	+
Scopulariopsis chartarum	-	+	-	+	-	+	-	-	-	-
Scopulariopsis sp.	-	+	-	-	+	-	+	-	+	-
Streptomyces sp	+	+	+	+	+	+	+	+	+	-

- = Negative growth

+ = Growth

Table : 3a pH measurement of different agar media after the growth of isolated fungi which appeared from the direct inoculation of straw pieces and incubated for ten days at 30^{0}C.

Different agar media used for inoculation		Each medium represented twice for two different pH's	
		Initial pH before inoculation	Final pH after inoculation
Corn meal agar	I	7.70	7.95
	2	9.00	7.55
Glucose- starch agar	I	7.70	6.40
	2	9.00	7.45
Malt agar	I	7.70	7.15
	2	8.60	6.75
Potato dext-rose agar	I	7.70	6.40
	2	9.00	5.50
Glucose- cel-lulose agar	I	7.70	7.27
	2	9.00	7.80

The same group of fungi were again isolated as outlined in part 1), though the growth conditions were still under alkaline pH's, but more frequent isolation of Alternaria tenius, Cephalosporium sp and Fusarium spp was noticed on these agars. This has further suggested that these isolates were quite capable of resisting a high pH, but were not active enough to grow frequently under the growth condition provided in Part 1). In addition, more fungi were observed and isolated which were not revealed in the previous part (see Table 1 for part 1).

Among the new isolates the most abundant one was Aureobasidium pullulans. It is interesting to note that in part 1) only Rhizopus sp was observed but more phycomycetes were isolated here such as Cunninghamella elegans and Mucor sp.

pH measurement during the fungal isolation period was also carried out and Table 2a reveals that most of the inoculated agars were still alkaline.

However, there is a possibility that some slow growing indigeneous fungi were capable of tolerating high pH's during their growth but could not be seen here due to the presence of fast growing fungi which had overgrown them. Walker (1941) has also suggested that the direct inoculation method tends to discriminate against slower growing fungi.

2.3.2. The fungi isolated from inoculation of washed straw

The fungi isolated by plating out washed straw on mineral salt agar supplemented with ammonia are given in Tables 4I, II, III, IV, V and VI.

Although no frequency of occurrence for isolated fungi was recorded in this technique, four types of isolates

Table 4I Fungi isolated from washed straw pieces inoculated onto mineral salt agar supplemented with 1% aqueous ammonia, after two weeks incubation at 30°C.

FUNGI	Isolation of fungi from the inoculation of straw pieces after six successive washings											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd
Aspergillus fumigatus	+	+	-	-	-	-	-	-	-	-	-	-
Aspergillus sp.	-	-	-	-	-	-	+	-	-	-	-	+
Chaetomium globosum	+	-	-	-	-	-	-	-	-	-	-	-
Chaetomium sp.	-	-	-	-	-	+	-	-	-	-	-	-
Coprinus cinereus	-	+	-	+	+	+	-	-	+	-	-	+
Coprinus sp.	-	-	-	-	-	-	-	-	-	-	+	-
Scopulariopsis brevicaulis	-	-	-	-	-	-	-	+	-	-	-	-
Scopulariopsis sp.	-	-	+	-	-	-	-	-	-	+	-	-

Table 4 II Fungi isolated from weashed straw pieces inoculated onto mineral salt agar supplemented with 0.5% aqueous ammonia, at 30°C

FUNGI	Isolation of fungi from the incoulation of straw pieces after six successive washings from two weeks incubation											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd	week 1st	2nd
Alternaria tenuis	-	-	-	-	-	-	-	+	-	-	-	-
Aspergillus fumigatus	+	+	-	-	-	-	-	-	-	+	-	-
Chaetomium globosum	+	-	-	-	+	-	-	-	-	-	-	-
Coprinus cinereus	+	+	-	+	-	+	+	+	+	+	+	+
Fusarium sp.	-	-	-	-	-	-	-	-	-	-	-	+
Scopulariopsis brevicaulis	-	-	+	-	+	+	-	-	-	-	-	-
Scopulariopsis sp	-	-	+	+	-	-	-	-	-	-	+	-
Streptomyces sp.	-	-	-	-	-	+	+	+	-	-	-	-

Table 4 III Fungi isolated from washed straw pieces inoculated onto mineral salt agar supplemented with 0.25% aqueous ammonia, at 30°C

FUNGI	Isolation of fungi from the inoculation of straw pieces after six successive washings from two weeks' incubation											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week
Alternaria tenuis	-	-	-	-	-	+	-	-	-	-	-	-
Aspergillus fumigatus	-	-	+	+	-	-	+	-	+	-	+	+
Aspergillus sp.	-	-	-	-	-	+	-	-	-	-	-	-
Chaetomium globosum	-	+	-	-	-	-	-	-	-	-	-	-
Coprinus cinereus	+	+	-	+	+	-	-	-	+	+	+	-
Coprinus sp.	-	-	+	-	-	-	-	+	-	-	-	-
Cephalosporium sp.	-	-	-	-	-	-	-	-	-	-	-	+
Fusarium sp.	-	-	-	-	-	-	-	+	-	-	-	-
Scopulariopsis brevicaulis	-	-	-	-	+	-	-	-	-	+	-	-
Scopulariopsis sp.	-	+	-	-	-	-	-	-	-	-	-	-
Streptomyces sp.	-	-	-	+	-	-	-	-	-	+	-	-

Table 4IV Fungi isolated from washed straw pieces inoculated onto mineral salt agar supplemented with 0.125% aqueous ammonia at 30°C

FUNGI	Isolation of fungi from the inoculation of straw pieces after six successive washings from two weeks incubation											
	1st washing week		2nd washing week		3rd washing week		4th washing week		5th washing week		6th washing week	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Alternaria tenuis	-	-	-	-	-	-	-	-	-	+	-	-
Aspergillus fumigatus	-	-	+	+	-	-	-	-	-	-	-	+
Chaetomium globosum	+	-	-	-	-	-	-	-	-	-	-	-
Coprinus cinereus	+	+	-	+	-	-	-	+	-	-	+	-
Coprinus sp.	-	-	-	-	-	+	-	-	-	-	-	-
Fusarium sp.	-	+	-	-	-	+	-	-	-	-	-	-
Scopulariopsis brevicaulis	-	-	-	-	+	+	-	+	+	+	-	-
Scopulariopsis sp.	+	-	-	-	-	-	+	-	-	-	-	-
Streptomyces sp.	+	+	-	-	+	-	-	-	-	-	-	-

Table 4V Fungi isolated from washed straw pieces inoculated onto mineral salt agar supplemented with 0.0625% aqueous ammonia at 30°C

FUNGI	Isolation of fungi from the inoculation of straw pieces after six successive washings from two weeks' incubation at 30°C											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week	1st week	2nd week
Alternaria tenuis	+	+	-	-	+	-	+	+	-	-	+	+
Alternaria sp	-	-	-	-	-	+	-	+	-	+	-	-
Aspergillus fumigatus	-	+	-	-	-	+	-	-	-	-	-	-
Aspergillus sp	-	-	-	-	-	-	-	+	-	-	-	-
Chaetomium globosum	-	+	+	-	-	-	-	-	-	-	-	-
Coprinus cinereus	+	+	+	+	+	+	+	+	-	+	+	+
Cephalosporium sp	-	-	-	-	-	-	-	-	-	+	+	+
Fusarium sp	-	-	-	+	-	-	-	+	+	+	-	-
Scopulariopsis chartarum	-	-	-	-	-	-	-	-	+	-	-	-
Scopulariopsis brevicaulis	+	-	+	-	-	-	-	-	-	-	-	-
Scopulariopsis sp.	-	-	-	+	-	-	-	-	-	-	-	-
Streptomyces sp	+	-	-	-	+	-	-	+	-	-	-	-

Table 4.VI pH Measurement of mineral salt agar media (supplemented with different percentages of aqueous ammonia) after the isolation of fungi, which appeared from the inoculation of washed straw pieces after two weeks incubation (first week = 1, second week = 2) at 30°C

Percentages of aqueous ammonia and initial pH of agar media		Final pH measurement of agar media plates which were inoculated with six successively washed straw pieces for the isolation of fungi											
% of NH_3	Initial pH	After 1st washing		After 2nd washing		After 3rd washing		After 4th washing		After 5th washing		After 6th washing	
		1	2	1	2	1	2	1	2	1	2	1	2
1%	10.10	8.72	7.88	8.67	8.80	8.57	8.20	8.70	8.44	8.67	8.40	8.57	8.40
0.5%	9.80	8.40	8.00	8.42	8.40	8.32	8.10	8.42	7.90	8.32	8.20	8.40	8.00
0.25%	9.00	8.37	8.20	8.80	8.25	8.60	7.70	8.40	8.00	8.60	8.15	8.60	8.10
0.125%	8.60	8.55	8.20	8.50	8.50	8.20	8.20	8.70	8.30	8.70	8.20	8.60	8.30
0.0625%	7.88	7.45	7.10	7.88	7.30	8.00	7.90	6.99	7.20	7.77	7.40	8.20	7.60

were found to dominate in all ammonia concentrated agar media including all successive serial washing techniques. These were: Aspergillus fumigatus, Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp. The fungus Chaetomium globosum was found occasionally, in spite of the fact that this was found abundantly by the direct inoculation method on the same media as used in this technique; thus suggesting that the washing technique had caused its removal from straw pieces.

It is noticeable that the gradual increase in the appearance of Alternaria tenuis, Fusarium sp. and Cephalosporium sp. was found up to 0.0625% ammonia concentrated agar. This indicated that the dominant inhabitants on this straw are the same as indicated by several workers previously (Waker, 1941; Gordon, 1944; Hyde and Galleymore 1951; Webster, 1957; Hudson and Webster, 1958; and Chang and Hudson, 1967).

No isolate of phycomycetes was observed from this technique, but was occasionally reported in the previous direct inoculation method (see 2.2.1.1.). This suggested that the washing techniques have only provided the isolation of particular groups of fungi which were presumably part of the indigenous flora on straw. Nevertheless, one has to bear in mind the fact that though this technique permitted the isolation of slow growing fungi, it still had restrictive conditions, and only those fungi able to tolerate different ammonia percentages and which used straw components as the sole carbon source could survive.

2.3.3. Inoculation of washings from straw

Tables 5I, II, III and IV summarised the results for isolation of fungi from washings of straw. Most of the fungi were not seen at all after inoculation of washed straw pieces, or were merely reported occasionally after the direct inoculation method. They are now, however, re-appearing after inoculating the agar plates with the water in which the straw was washed.

Among different isolates obtained, the most abundant one seemed to be the fungus Auriobasidium pullulans, which appeared on all agar media used in this experiment. In addition, Penicillium spp were also found to be competing frequently with the said species along with the appearance of Aspergillus fumigatus and other Aspergillus spp. However, the presence of Chaetomium globosum also revealed itself frequently on agar plates of nearly all inoculated serial washings. This may provide the reason for these species being rarely isolated during serial inoculation of washed straw, as these species mostly seemed to be washed away during serial washing and were collected in the washings. The other frequent isolates observed were Alternaria tenuis, Cephalosporium sp. and Fusarium spp. Their appearance was seen on early serial washings, but seemed to disappear from agar plates as inocula from the lower washings were plated out. However, this still showed their predominant colonization capacities on straw. However, similar characteristics were shown by Scopulariopsis brevicaulis and Streptomyces sp. which showed their abundant appearance on washing inoculation.

It is obvious from Tables 5I, II and III that the appearance of fungal isolates was found to be gradually

Table 5.1. Fungi isolated from washings of straw inoculated onto glucose-cellulose agar at 30°C for two weeks and pH 9.0 (2)

FUNGI	Fungi isolated from six successive washings inoculated onto agars at pH 7.7 (1) and pH 9.0 (2)											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2
Alternaria tenuis	+	+	+	-	-	+	-	-	-	-	-	-
Alternaria sp	-	-	-	+	-	-	-	-	-	-	-	-
Aspergillus fumigatus	-	-	-	-	+	-	-	-	-	-	-	-
A. flavus	-	-	-	-	+	-	-	-	-	-	-	-
Aspergillus sp	+	-	-	-	-	-	-	-	-	-	-	+
Arthobotrys sp												
Aureobasidium pullulans	-	-	-	-	+	-	+	-	-	-	-	-
Cephalosporium sp	+	-	+	+	+	-	-	-	-	-	-	-
Chaetomium globosum	+	+	+	-	+	-	-	-	+	-	-	-
Chaetomium sp	+	+	+	-	-	-	+	-	-	-	-	-
Fusarium spp	+	-	+	-	-	-	-	-	-	-	-	-
Paecilomyces sp	-	+	-	-	-	-	-	-	-	-	-	-
Penicillium spp	+	-	+	-	-	-	+	+	-	+	-	+

Table 5.II Fungi isolated from washings of straw inoculated onto glucose- starch agar at 30°C for ten days

FUNGI	Fungi isolated from six successive washings of straw inoculated onto glucose-starch agars at pH 7.70 (1) and pH 8.60 (2)											
	1st washing pH:1	pH:2	2nd washing pH:1	pH:2	3rd washing pH:1	pH:2	4th washing pH:1	pH:2	5th washing pH:1	pH:2	6th washing pH:1	pH:2
Alternaria tenuis	+	-	-	+	-	+	-	-	-	-	-	-
Alternaria sp	-	+	-	-	-	-	-	-	-	-	-	-
Aspergillus fumigatus	-	+	-	+	+	-	+	+	+	-	-	-
A. niger	-	-	+	-	-	-	-	-	-	-	-	-
Aspergillus sp	+	+	+	-	-	-	-	-	-	-	+	-
Aureobasidium pullulans	+	+	-	+	-	+	+	-	+	+	+	+
Botrytis cineria	-	-	+	-	-	-	-	-	-	-	-	-
Cephalosporium sp	+	-	-	-	-	-	-	-	-	-	-	-
Chaetomium globosum	+	+	-	+	-	-	-	-	-	-	-	-
Chaetomium sp	+	-	-	+	-	-	-	-	-	-	-	-
Fusarium spp	-	+	-	+	-	+	-	-	-	-	-	-
Cliocladium roseum	+	-	-	-	-	-	-	-	-	-	-	-
Mucor sp	-	+	-	-	-	-	-	-	-	-	-	-
Penicillium spp	-	-,	-	+	-	+	+	+	-	-	-	-
Rhizopus sp	-	-	+	-	-	-	-	-	-	-	-	-
Scopulariopsis brevicaulis	-	-	-	+	+	-	-	-	-	+	-	-
S. chartarum	-	-	-	-	-	+	-	-	-	-	-	-
Scopulariopsis sp	-	+	-	-	-	-	-	-	-	-	-	-
Streptomyces sp	-	-	-	-	+	-	+	-	-	-	-	-
Trichoderma viride	-	-	+	-	-	-	-	-	-	-	-	-

Table 5.III Fungi isolated from washing of straw inoculated onto potato dextrose agar at 30°C for ten days

FUNGI	Fungi isolated from washings of straw inoculated onto potato dextrose agars at pH 7.70 (1) and pH 9.00 (2)											
	1st washing		2nd washing		3rd washing		4th washing		5th washing		6th washing	
	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2	pH:1	pH:2
Alternaria tenuis	+	+	+	+	-	+	-	-	-	-	-	-
Alternaria sp	-	-	+	-	-	-	-	-	-	-	-	-
Aspergillus fumigatus	+	+	+	-	+	+	+	-	-	-	-	-
A. flavus	-	-	-	-	+	-	-	-	-	-	-	-
A. niger	-	-	+	-	-	-	-	-	-	-	-	-
A. versicolor	+	-	-	-	-	-	-	-	-	-	-	-
Aspergillus sp	+	+	+	+	-	-	-	-	-	-	-	-
Arthobotrys sp	+	-	-	-	-	-	-	-	-	-	-	-
Aureobasidium pullulans	+	+	-	+	+	+	-	+	+	+	+	+
Botrytis sp	-	-	-	+	-	-	+	-	-	-	-	-
Cephalosporium sp	-	-	-	-	-	+	+	-	-	-	-	-
Chaetomium globosum	+	+	-	+	-	-	-	+	-	-	-	-
Chaetomium sp	-	-	-	+	-	-	-	-	-	-	-	-
Fusarium spp	+	+	+	-	-	-	-	-	-	-	-	-
Paecilomyces sp	-	-	-	-	-	+	-	-	-	-	-	-
Penicillium spp	-	-	+	+	-	-	-	+	+	-	-	-
Scopulariopsis brevicaulis	-	+	+	-	+	-	+	-	-	-	-	-
S. chartarum	-	-	-	-	-	+	-	-	-	-	-	-
Streptomyces sp	-	+	-	+	-	-	-	-	-	-	-	-
Trichoderma viride	-	-	-	+	-	-	-	-	-	-	-	-

Table 5.IV. pH measurement of different agar media after fungal isolation from the inoculation of washings of straw, after 10 days of incubation at 30°C

Inoculation of washing of straw onto agar media adjusted at different pH'		Final pH of different agar media after the inoculation of six successive washings from straw					
Agar media	Initial pH	Number of washing					
		1st	2nd	3rd	4th	5th	6th
Glucose-cellulose agar	7.7	7.55	7.45	7.55	7.95	7.40	7.35
	9.0	8.17	7.7	8.4	8.47	8.3	7.7
Glucose-starch agar	7.7	5.30	5.40	5.90	6.10	5.80	6.00
	8.6	7.00	6.20	6.7	7.10	7.70	7.40
Potato dextrose agar	7.7	5.40	5.60	6.00	5.90	6.30	6.80
	9.0	6.20	6.40	6.80	7.10	7.30	7.20

decreasing after systematic inoculation of serial washings. Maximum isolates were found on agar inoculated with the first washings whereas the least number was noticed on plates inoculated with the 6th washing. This shows the gradual detachment of fungal propagules from straw pieces while serial washing was in operation. (Harley and Waid, 1955).

After seeing the same species of fungi even after the inoculation of the 6th washing, one could probably assume that it would be possible to isolate the same species after plating out of further washings.

2.3.4. Fungi from ammonia treated straw

Table 6 shows those fungi observed microscopically from ammonia treated straw and Tables 7a and b show their isolation on agar plates.

Microscopic examination of ammonia treated, incubated straw revealed the presence of the same fungi which were reported from washed straw on agar plates (see in section 2.2.1.3).However, the appearance of *Aureobasidium* sp was also observed quite often along with the occasional appearance of *Graphium* sp. and *Penicillium* spp with few fungal species which were difficult to recognize under the microscope. However, only one species of *Rhizopus* was seen.

Mostly the same groups of fungi which were revealed during microscopic studies were isolated after plating out of ammonia treated, incubated straw pieces. However, a few more additional fungi were noticed by the appearance of *Botryotrichum piluliferum*, *Botrytis cineria* and *Cunninghamella elegans*. Nevertheless, their isolation was occasional. Moreover, the isolation of *Graphium* sp was not seen on any inoculated agar, in spite of the fact that it was viewed under the microscope.

Table 6. Microscopic examination of aqueous ammonia treated straw samples

FUNGI	Fungi observed under the microscope from the straw pieces which were previously amended with different aqueous ammonia and incubated for two weeks at 30°C									
	1%		0.5%		0.25%		0.125%		0.0625%	
Fungi	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Alternaria spp.	-	-	+	+	+	+	+	+	+	+
Aspergillus spp.	+	+	+	+	+	+	+	+	+	+
Aureobasidium sp.	-	-	-	-	+	+	+	+	+	+
Chaetomium spp.	+	+	+	+	+	+	+	+	+	+
Coprinus sp.	+	+	+	+	+	+	+	+	+	+
Botrytis sp.	-	-	-	-	-	-	-	-	+	-
Fusarium spp.	-	-	-	+	+	-	+	-	+	+
Graphium sp.	-	-	-	-	-	-	-	-	+	-
Penicillium sp.	-	-	-	-	-	+	-	-	-	-
Rhizopus sp.	-	-	-	-	-	-	+	-	+	-
Scopulariopsis spp.	+	+	+	+	+	+	+	+	+	+
Streptomyces sp.	-	+	+	+	+	+	+	+	+	+

- = Negative sign indicates that fungus was not viewed

+ = Positive sign indicates that fungus was viewed microscopically

1st = One week of incubation

2nd = Two weeks of incubation

Table 7a Fungal isolation on agar from one week ammonia treated straw at 30°C

FUNGI	Isolation of fungi from straw (which had previously been treated with different aqueous ammonia supplemented mineral salt solution, after two weeks' incubation									
	1%		0.5%		0.25%		0.125%		0.0625%	
	1	2	1	2	1	2	1	2	1	2
Alternaria tenuis	-	-	-	+	+	+	+	+	+	+
Alternaria sp	-	-	-	-	-	-	-	+	-	-
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+
Aspergillus spp	-	-	+	+	+	-	+	+	+	+
Aureobasidium pullulans	-	-	-	-	-	+	-	-	+	+
Auriobasidium sp.	-	-	-	-	-	+	-	-	-	-
Botrytis cineria	-	-	-	-	-	+	-	-	-	-
Botryotrichum piluliferum	-	-	+	-	-	+	-	-	-	-
Cephalosporium sp.	-	-	-	-	-	+	-	+	+	-
Chaetomium globosum	+	+	+	+	+	+	+	+	+	+
Chaetomium sp.	-	-	-	-	+	-	-	-	+	-
Coprinus cinereus	+	+	+	+	+	+	+	+	+	+
Cunninghamella elegans	-	-	-	-	-	-	+	-	-	-
Fusarium spp.	-	-	-	+	+	-	-	+	-	-
Paecilomyces sp.	-	-	-	-	-	+	-	-	-	-
Penicillium sp.	-	-	-	-	-	+	+	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	+	-	-

Table 7b. Fungal isolation from two weeks aqueous ammonia treated incubated straw, at 30°C

FUNGI	Isolation of fungi from straw (which had been previously treated with different ammonia supplemented mineral salt solutions) after two weeks incubation									
	1%		0.5%		0.25%		0.125%		0.0625%	
	1st	2nd weeks	1st	2nd weeks	1st	2nd weeks	1st	2nd weeks	1st	2nd weeks
Alternaria tenuis	-	-	-	+	+	+	+	+	+	+
Alternaria sp.	-	-	+	-	-	-	-	-	-	-
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+
Aspergillus sp.	+	+	-	-	-	-	-	-	-	+
A. versicolor	-	-	-	-	-	+	+	-	+	-
Aureobasidium pullulans	-	-	-	-	+	+	+	+	+	+
Botryotrochum piluliferum	-	-	-	-	-	-	-	+	-	-
Cephalosporium sp	-	-	-	-	-	+	+	+	+	+
Chaetomium globosum	+	+	+	+	+	+	+	+	+	+
Chaetomium sp.	-	-	-	-	+	-	-	-	-	-
Coprinus cinereus	+	+	+	+	+	-	-	+	+	+
Fusarium spp.	-	-	+	+	+	-	-	+	-	+
Penicillium spp.	-	-	-	-	-	-	+	-	+	-
Scopulariopsis brevicaulis	+	+	+	+	-	-	+	+	-	+
Scopulariopsis sp.	-	-	-	-	-	+	-	-	+	-
Streptomyces sp.	+	+	-	-	+	+	-	-	-	+

2.4. DISCUSSION

The results illustrate the isolation of different groups of fungi from barley straw. Their presence on straw was as a result of their being predominant colonizers or resulting from the common air spora from the surrounding atmosphere. The isolation techniques revealed the appearance of fungi which were quite capable of growth even under highly alkaline pH conditions. Among these isolates, the most dominant ones were: Aspergillus fumigatus, Botryotrichum piluliferum, Chaetomium globosum, Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp.

No attempt was made at this level to measure the mycelial growth of all isolated fungi by growing them separately under the influence of high pH conditions. However, in spite of that, an attempt was made to subculture the isolated fungi individually on alkaline pH media for pure culture studies and to maintain them under the said conditions throughout the isolation period. This criterion was used to check that the isolated fungi could actually survive and grow individually under high pH conditions.

Having seen the results from the direct inoculation of straw pieces, one could easily point out the difference in the enumeration of fungal isolates on two different sets of agar i) and ii). (See Tables 1 and 2). The purpose of the direct inoculation method was to encourage the isolation of both the surface fungal propagules and the predominant fungal flora from straw. Nevertheless, it could be seen that most of the surface propagules did not come up on agar where the plates were supplemented with ammonia, and the only carbon source available was in the form

of suspended straw peices. This could easily be revealed by the appearance of different groups of phycomycetes such as: Cunninghamella elegans, Mucor sp. and Rhizopus sp on the plates of corn meal agar, czapex dox agar, malt agar, glucose and cellulose agar and potato dextrose agar, i.e. ii set of agar media (See Table 2). However, hardly any of these fungi (except Rhizopus sp) were seen on agar plates supplemented with ammonia (see Table 1). This difference could also be seen among other groups of fungi as the appearance of fungi imperfecti, e.g. Alternaria tenuis, Aspergillus fumigates, Aspergillus versicolor, Aspergillus spp. Aureobasidium pullulans, Botrytis cineria, Cephalosporium sp., Fusarium sp., Penicillium spp., and Scopulariopsis brevicaulis were noticed more frequently on the second set of agar media. Nevertheless, except Aspergillus fumigatus and Scopulariopsis brevicaulis, the occasional appearance of only Alternaria tenuis, Cephalisporium sp. and Fusarium sp. were seen on the first set of agar media. This showed the inability of the above fungi to grow either under the influence of the first set of media or to grow under the influence of incorporated ammonia or due to their inability to use suspended straw components as the only carbon source. In addition to that, there may be one more good reason that, although both sets of agar media have provided the alkaline pH conditions, a greater alkaline range was obtained with the agar where different ammonia percentages (1, 0.5, 0.25, 0.125 and 0.0625%) were incorporated. This situation probably did not permit the isolation of fungi which could only tolerate moderate alkaline pH conditions in their growth environment. However, the Basidiomycete Coprinus cinereus has shown its

frequent isolation on all agar media provided for direct inoculation. This fungal isolate was found to be highly alkalophilic in nature and has been reported to be growing very well in the presence of aqueous ammonia (Fries, 1955, 1956; Seal, 1973; Sagara, 1975; Penn, 1976).

To see the actual difference with the direct inoculation method, a serial washing technique was employed (Harley and Waid, 1955) before inoculating the straw pieces onto agar media. However, the same ammonia supplemented agar media were used as reported earlier, and, having done this, the same group of isolates were revealed as reported in Table 1.

Throughout the examination of fungal isolates, the most dominant ones were found to be _Aspergillus fumigatus_, _Coprinus cinereus_, _Scopulariopsis brevicaulis_ and _Streptomyces_ sp. During study of the fungi of wheat straw compost, Chang and Hudson (1967) have also carried out the washing of straw pieces before inoculating on agar plates. They also reported the isolation of different _Coprinus_ species, and among those the most common ones were found to be _C. cinereus_ and _C. megacephalus_. Eastwood (1952) failed to isolate _Coprinus_ species from dilution plates while studying the fungal flora of barley straw compost. However, she has reported the appearance of sporophores of _Coprinus lagopus_ from her composting straw.

No appearance of fresh fungal isolates were revealed when inoculation of washings from straw was made on agar plates ; as most of the detached fungal propagules from

straw washings have been seen isolated on agar (see Tables 51, II and III). However, the most abundant appearance of Aureobasidium pullulans was found to be during inoculation on all agar media. However, this fungal isolate was previously reported as a primary coloniser on straw and grasses (Webster, 1956, 1957; Hudson and Webster, 1958).

Similar results were obtained in comparison to direct inoculation when ammonia treated straw pieces were inoculated on cellulose agar (Eggins and Pugh, 1962). This again showed the inability of most of the fungi to grow in the presence of ammonia. Furthermore, this technique has provided the most appropriate information that most of the fungi appearing throughout this isolation programme could probably grow under the influence of alkaline pH ranges, but presumably could not tolerate the presence of ammonia in their growth environment. However, the appearance of Coprinus cinereus, Chaetomium globosum, Aspergillus fumigatus, Scopulariopsis brevicaulis and Streptomyces sp. were again abundant with the ammonia treated straw inoculation technique. A fresh species, Botryotrichum piluliferum, was also revealed for the first time by this technique. This species was originally isolated from manure and from excreta of deer and goats (Saccardo, P.A.E., Marchal, 1885; Rostrup, O. 1916). According to the suggestion made by previous workers, a selective method was required for its isolation, e.g. soil plates with cellulose agar (Pugh, Blackeman, Morgan-Jones and Eggins, 1963).

A preliminary investigation of the isolated fungi revealed that most of them were found to be highly cellulosic even under highly alkaline conditions, e.g. Chaetomium

globosum, Coprinus cinereus, Botryotrichum piluliferm. However, a detailed study on the utilization of various carbon sources was undertaken in the proceeding chapter (see Chapter 5), and the final selection was made from these i.e. the most active fungi were chosen as the agents for utilizing in the biological up-grading process of straw.

CHAPTER 3

Isolation of *Coprini* from a range of vegetation

CHAPTER 3

3.1. INTRODUCTION

The work presented in this part of the investigation was an attempt to isolate *Coprinus* species from different vegetation, collected from various urban and suburban locations of Birmingham, England, during late August and early September, 1977.

During fungal isolation from barley straw as reported in Chapter 2, a predominant colonization of *Corpinus* species, particularly *C. cinereus*, was reported from most of the inoculated agar plates. This suggested the idea that there should be a possibility of the presence of *Coprinus* species on other vegetation as well, and it should be worthwhile to investigate its presence on these.

Before going to any further detailed study on the genus *Coprinus*, it is worth mentioning here that there was a great deal of confusion in the past over the correct nomenclature of the *Coprini*. Pinto-Lopes (1971) has provided a detailed survey, which shows that if one could consider *C.cinereus* as an example in particular then it could be seen that this species has been given many synomyms by a number of workers which include: *C. fimetarius*, (Buller, 1931); *C. lagopus*, (Anderson, 1969; Casselton and Lewis, 1967: Day and Anderson, 1961; Day and Roberts, 1968, and Eastwood, 1952); *C. stercorarius* (Kniep, 1920, and Mounce, 1921) and *C.macrorhizus* (Buller, 1924 and Dickson, 1936).

For the identification of a number of *Coprinus* species a coursework was organised by Kemple and Watling (1977) under the heading of *Coprinus* Workshop. This work has also provided the criterion for differentiating *C. cinereus* from

C. lagopus.

The technique used for isolation was adapted from one employed for inoculation of ammonia treated straw as mentioned in Chapter 2, section 2.2.4. However, 1% ammonia supplemented mineral salt solution was incorporated into BS438 medicine bottles containing uniformly sized, cut, collected vegetation (Twigs). Having been incubated at 30°C for a required period of time, the isolated Basidiomycetes mycelia were subcultured onto agar plates and a further inoculation was made for pure culture study. The identification of Coprinus species was made after seeing the appearance of mature fruiting bodies.

3.1.1. Coprinus species

The fungus Coprinus was also considered as coprophilous. This is due to the fact that most of the Coprinus species come up on the dung of herbivourous animals. However, according to the information received by Buller (1931) in his series of investigations on Researches on Fungi, the first reference for the existence of the genus Coprinus was found to be in the statement of Theophrastus, circa 300 B.C. where he indicates that "the fungus which grow on dung have no bad smell". However, Vaillant in the year 1727, has reported a species of Coprinus stramentarius, by giving a detailed account and also some appreciable copperplate illustrations of it (Buller, 1922).

3.1.2. First microscopic examination of Coprinus spores

It is interesting to note that the first microscopic examination of Coprinus spores was made by a Florentine botanist, Micheli (1729), who had differentiated some species after examining them under the microscope and among these species, Coprinus macrohizus and Coprinus sterquilinus were common (Buller, 1924).

3.1.3. Coprophilous fungi

Literally coprophilous are those organisms which can live on dung or excrement of herbivorous animals, viz. camels, cattle, geese, giraffes, goats, horses, rabbits, and sheep, etc. Dung or faecal pellets excreted by herbivores are very rich in nutrients. This is however, due to the presence of an excessive amount of undigested organic materials, i.e. cellulose, hemicellulose and lignin, etc; availability of a high percentage of nitrogen source and the presence of a rich amount of vitamins - particularly a growth factor called coprogen. (Hasseltine Whitehill, Pidocks, Tenhagen, Bohonos, Hutchings and Williams, 1953).

The easily available nutritional requirements and a high percentage of water, i.e. 59-90% (Morrison, 1959; Nicholson,

1966), in the dung provides a very good habitat for the establishment and growth of a large number of micro-organisms, such as: bacteria, actinomycetes, myxomycetes, protozoa, moluscs and nematode. However, hitherto, the literature indicates that most of the previous workers in this field have shown their interest by studying the fungal succession pattern on deposited dung or faecal pellets. Here it is important to note that the presence of *Coprinus* species were mostly indicated by several investigators concerned in succession studies. Carter (1959) has reported the isolation of *C. heptemerus*, from rabbit pellets, whereas Harper and Webster (1964) have indicated the presence of *C.pseudoradiatus* from the same type of substrate. Nevertheless, Rege (1927), who did not study fungal succession, reported the isolation of *Coprinus* species from a heap of decomposed horse manure. In addition to that, Fries (1955) collected some coprophilous *Coprinus*, from different habitats, such as: *C. fimetarius*, from new manure, *C. ephemerus* from stale manure, *C. atramentarius* and *C.plicatilis* from fruit bodies, from old lawns or meadows. Recently, a Japanese worker, Sagara, N. (1975) has reported the isolation of a number of *Coprinus* species from the ammonia treated soil in Japan.

3.1.4. Agents Responsible for Spore Dissemination of Coprophilous Fungi

Buller, (1933), has suggested that the coprophilous fungi can be divided into two groups on the basis of their spore dispersal. According to his statement, these groups are:

1) a more primitive group which successively makes use of three external agents - the wind, flowering plants and

herbivorous animals;

ii) A dispersion made relying on the wind, and which successively makes use of only two external agents - flowering plants and herbivorous animals. However, it is interesting to note that the spore dispersion of some *Coprinus* species was also achieved in one of the first ways stated by Buller.

3.1.4.1. Role of the wind

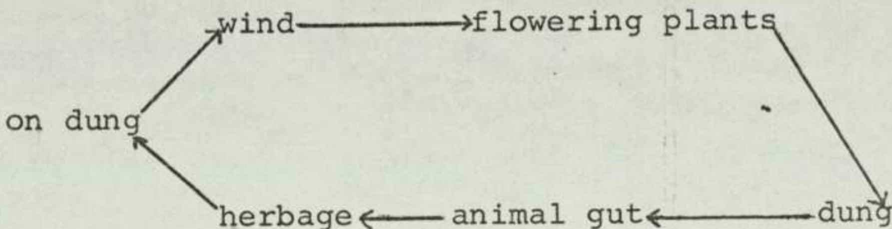
The wind plays a very important role in spore dissemination of Coprophilous fungi. According to Buller (1933), the first group of fungi disseminate their spores simply through the agency of wind, and thus the spores may be deposited on different habitats, viz:
on herbage, in pasture, in pools, lakes, streams, seas, on rocky ground, the bare earth, the leaves of trees, etc. It is noteworthy that the early work of Buller (1909) has already indicated that the *Coprinus spp* were found to be wind-borne in nature. Nevertheless, Massee (1906) had a different viewpoint on *Coprinus* spore dispersion. He suggested that the spore dispersion takes place by deliquescence of the mature gills and drops to the herbage in pasture in liquid form which carries a large number of spores along with it.

The second group of fungi discharge their spores in the form of large masses. This helps them to stick onto or attach very quickly to the surrounding vegetations, from these they drop down to herbage and settle on it, where other agents effect the further dissemination of laden spores.

3.1.4.2. Role of herbivorous animals

Herbivorous animals grazing on herbage are likely to swallow the laden spores previously deposited by Coprophilous fungi. However, the ingested spores pass through the digestive tract of the animal without being damaged during the process of digestion, and finally drop in deposited dung or faecal pellets. Reports of some researchers have showed that the period of dormancy for spore germination declines by the effect of intestinal temperature and with the action of various digestive juices or enzymes. These pre-treatments presumably give stimulating support for rapid germination and fructification of spores after their deposition (Massee and Salmon, 1902; Dodge, 1912; Ingold, 1953; Harper and Webster, 1964 and Larsen, 1971).

It was suggested by previous workers that the spore dispersion cycle took the following form:



This shows that the herbivorous animals are acting as the active agents for spore transportation and distribution, as they can possibly transport the spores for a distance of some miles while grazing or moving to new areas of land and discharging their faecal deposit.

However, some other factors are also relevant to the dissemination process. These include splashes of water, spraying of animal dung or slurry into cultivates areas of land; and from the site of compost heaps etc. (Eastwood,

1952; Chang and Hudson, (1967)

Several investigators have tentatively isolated a large number of coprophilous fungi along with *Coprinus* spp from various agricultural waste products. Eastwood (1952) has given an account of appearance of sporophores of *C. lagopus* from the composting of cut barley straw. An investigation by Chang and Hudson (1967), on the other hand, revealed the presence of some *Coprinus* spp such as *C. cinereus* and *C. megacephalus* on wheat straw compost.

3.1.5. Some factors promoting the growth of Coprinus species

Earlier studies on *Coprinus* have revealed the influence of some environmental factors responsible for promoting mycelial development. Among these factors, the role of pH and temperature are very important, along with the effects of nitrogen sources.

Literature on *Coprinus* reveals that these fungi can grow quite happily under alkaline conditions. Johnson and Jones (1941) have found that the species of *Coprinus cf. cubensis* showed its normal growth activities at pH 5.9-9.2 when inoculated on agar plates. However, later on a series of investigative papers were published by Fries (1945, 1953, 1956, 1956) on Physiological studies of *Coprinus*.

Her investigation also indicated that this group of fungi were highly alkalophilic and grew in nature on manure or soil rich in humus. To satisfy this evidence she performed a series of experiments, to estimate the pH values of some samples from the habitat of different *Coprinus* species, as shown in Table 8. This table indicates that out of 8 different samples, only the habitat of *C.ephemeroides* and *C.*

Table : 8

pH measurement of some samples from the habitats of eight different Coprinus species

Serial number	Coprinus species	Number of investi-gated habi-tats	pH values
I	Coprinus atramentarius	2	7.8-8.I
2	Coprinus comatus	3	7.7-7.9
3	Coprinus ephemeroides	3	6.9-7.5
4	Coprinus ephemerus	2	7.0-8.7
5	Coprinus fimetarius	I	8.4
6	Coprinus micaceus	3	6.6-7.6
7	Coprinus nivacus	2	7.I-7.5
8	Coprinus radialus	I	8.4

Data provided by Fries (I956).

micaceus showed the pH values from slightly acid to aklaline ranges, whereas the rest of the values came within the alkalinic pH range.

3.1.6. Action of temperature on Coprinus species

Usually, the Basidiomycetes are classed among those groups of fungi which do not possess resitance against high temperature in their growth environments. On the contrary, the growth of Coprinus species were one of the exceptions, which can resist a high degree of temperature. Rege (1927) estimated the temperature optima for the growth of a Coprinus sp. which had been isolated from a heap of decomposed manure. He found that the isolate was grow quite well at 30-35°C. However, it is interesting to note that the growth was vegetative at this temperature, i.e. without showing any initial fruit body formation. It is noteworthy that a similar type of result was also obtained by Chang (1967), when she found that Coprinus cinereus (previously isolated from wheat straw compost) (see Chang and Hudson, 1967) showed extensive mycelial growth at 35°C in pure culture without showing any indication of the appearance of fertile sporophores at that temperature. However, detailed studies by Fries (1956) have evaluated the relation of temperature to the growth rates of different Coprinus species and out of seven different species, most of them showed their growth activities at 25-35°C and preferred 30°C for the optimal growth. Nevertheless, one species, i.e. C.fimetarius was found to show its temperature optima at 40°C and was still able to grow at 44°C. She further noticed that the growth

of this fungus could considerably increase if media were supplemented with a growth factor, i.e. methionine (Fries, 1953). On the basis of these investigations, she made a suggestion that the high temperature tolerant species of Coprinus were all coprophilic in nature

All the above outlined investigations have pointed out that the temperature tolerating abilities of Coprinus species were higher, except in the species of C. atramentarius and C. micaceus, which preferred to grow at 15-35°C, with maximum temperature up to 30°C as reported by Fries (1956). This has suggested the idea that one can quite happily choose a temperature, say 30°C, for isolation of these fungi from different habitats.

3.2. EXPERIMENTAL PROCEDURE

Twigs with flowers attached from different vegetations were cut with a sharp scalpel and collected under aseptic conditions from urban and suburban locations in Birmingham, England. The materials were collected during late August and early September, 1977, on a dry day (see Table 8 a)

Mineral salt solution with 1% added ammonia was prepared (see appendix) without any addition of a bacteriostatic substance. However, two other agar media were prepared for subculture and pure culture studies:
i) cellulose agar (Eggins and Pugh, 1962) using the same method as indicated in Chapter 2, section 2.2.1.1.; and
ii) Potato maltose agar (see appendix). The former agar was added with 30 mg/litre Rose bengal to suppress the growth of bacteria (Ottow, 1972).

Table 8a. Collection of summer vegetation from various locations of urban and suburban area in Birmingham

Family name of collected vegetation	Names of Species		Location of vegetation	Collection date
	Botanical	Common English		
Chenopodi-aceae	Atiplex patula	Common orache	Surrounding wasteland, near city centre	8.9.1977
	Chenopodium album	Fat hen	Surrounding wasteground, Handsworthwood	8.9.1977
	Polygonum persicaria	Common persicaria	Surrounding wasteground, Handsworthwood	8.9.1977
Compositae	Artmisia vulgaris	Mugwort	Surrounding wasteland, Stafford Road	8.9.1977
	Circium arvense	Creeping thistle	Surrounding car park, City centre	7.9.1977
	Seneciosqualidus	Oxford ragwort	Surrounding car park, City centre	7.9.1977
Gramminae	Agrostis tenuis	Common bet	Surrounding Langley Green Railway Stn.	8.7.1977
	Dactylis glomerata	Cocks foot	Surrounding Aston University	27.8.1977
	Milium effusum	Wood millet	Surrounding Langley Green Railway Stn.	27.8.1977
	Nardus stricta	Mat grass	Surrounding Aston University	26.7.1977
Onagraceae	Epilobium Angustifolum	Rose bay	Surrounding Aston University	27.8.1977
Papilion-aceae	Trifolium sp.	?	Surrounding car park, City centre.	27.8.1977
Resedaceae	Reseda luteola	Wild mignonette	Surrounding Aston University car park	7.9.1977
Rosaceae	Rubus sp.		Wasteland surrounding, Ladywood Road	7.8.1977

3.2.1. Isolation of fungus Coprinus from inoculated vegetations

A twig approximately 8 cm long (previously cut) from each collected vegetation was placed in sterile BS 838 flat shaped medicine bottles, then 1% ammonia concentrated mineral salt solution was added to each oontainer, using an amount which was sufficient to soak the material thoroughly. However, five replicates were used for each vegetation sample, the whole technique being employed under aseptic conditions.

The samples were incubated at 30°C for an appropriate period of time as required for the isolation of Basidiomycetes. Nevertheless, the main concern here was to isolate *Coprinus* species, and, according to Fries (1955), the said temperature was quite suitable for the growth of most of these species. During the incubation period, the bottles were viewed occasionally to check for any appearance of mycelial growth. After mycelium had been found growing on the surface of twigs, it was examined under the illuminated microscope for viewing the presence of clamp connections. This type of examination was an obvious method for identification of most of the Basidiomycetes (Nobles, 1948, 1964).

Once the presence of clamp connections had been viewed, the isolated mycelium from different vegetation was subcultured on the agar plates. This was carried out by cutting, aseptically, the small pieces of materials from the twigs - which had colonized with the mycelial strands. The pieces were inoculated onto the surface of cellulose agar (Eggins and Pugh, 1962), and pure culture studies were made by further sub-culture onto potato maltose agar (see appendix). The identification of isolated *Coprinus* species was carried

out after the appearance of the fruit bodies, while the plates were incubated into illuminated incubator which helps in the rapid formation of fruiting bodies (Kemple and Watling, 1977).

3.3. RESULTS AND DISCUSSION

The results for the isolation of Coprinus species from different vegetations are summarised in tables 9, 10 and 11. However, the appearance of other groups of fungi was also noticeable during the incubation of collected vegetation but their growth was gradually suppressed with the gradual increase in the mycelial appearance of Basidiomycetes which nearly covered the entire surface areas of most of the suspended twigs.

The fruit body formation from most of the growing mycelium of Coprinus species was not noticeable during the first 10 days of inoculation of twigs, but later on the initial fruit bodies were seen. Nevertheless, the emphasis was not really made at this level of isolation on studying fruit body formation. However, according to the statement made by Sagara (1975) "a chemoecological group of fungi which sequentially develop reproductive structures exclusively or relatively luxuriantly on the soil after a sudden addition of ammonia, some other nitrogenous materials which react as bases by themselves or on decomposition, or alkalis". The term 'chemoecological group' was used by Sagara to indicate ammonia fungi, which will be discussed in more detail later.

Ten out of fourteen inoculated vegetation samples showed the isolation of Coprinus species. Among those the most common one was found to be Coprinus cinereus, which was seen growing on five different sets of vegetation samples suspended in BS 838 medicine bottles.

No other techniques were employed in this part of the isolation programme except the direct inoculation method. For that reason it should be difficult at this stage for

Table 9. Indications of Coprini isolated from different vegetations at 30°C after ten days incubation period

Vegetations impregnated with 1% aqueous ammonia supplemented mineral salt solution	Indications for Coprini isolation		
	Growth of Basidio-mycete	Mycelia colon-isation	Fructi-fication
Atiplex patula	+	Exensive	+
Chenopodium album	-	NV	-
Polygonum persicaria	+	Moderate	+
Artmisia vulgaris	+	Extensive	+
Cirium arvense	+	Extensive	+
Senecio squalidus	+	Extensive	+
Agrostis tenuis	+	Fair	-
Dactylis glomerata	+	Extensive	+
Milium effusum	+	Extensive	+
Nardus stricta	+	Extensive	+
Epilobium angustifolum	+	Moderate	-
Trifolium sp.	-	NV	-
Reseda luteola	+	Fair	-
Rubus sp.	+	Extensive	+

NV = Not visible

Table : 10

Inoculation of Basidiomycete mycelium isolated from different vegetations at 30^{0}C. after ten days.

Collected Vegetation	Inoculation on agar medium			
	Sub-culture on cellulose agar		Pure culture inoculation on maltose agar	
	Sclerotia formation	Fructification	Sclerotia formation	Fructification
Atiplex patula	+	+	+	+
Chenopodium album	–	–	–	–
Polygonum persicaria	+	+	+	+
Artmisia vulgaris	+	+	+	+
Cirium arvense	–	+	–	+
Senecio squalidus	+	+	+	+
Agrostis tenuis	–	–	–	–
Dactylis glomerata	–	+	–	+
Milium effusum	+	+	+	+
Nardus stricta	+	+	+	+
Epilobium angustifolum	–	–	–	–
Trifolium sp.	–	–	–	–
Reseda luteola	–	–	–	–
Rubus sp.	+	+	+	+

Table : 11

Isolation of Coprinus species from different vegetations

Vegetations used for fungus Coprinus isolation	Coprini isolated
Atiplex patula	Coprinus cinereus
Chenopodium album	Nil
Polygonum persicaria	Coprinus sp.
Artmisia vulgaris	Coprinus cinereus
Cirium arvense	Coprinus lagopus
Senecio squalidus	Coprinus cinereus
Agrostis tenuis	Nil
Dactylis glomerata	Coprinus lagopus
Milium effusum	Coprinus cinereus
Nardus stricta	Coprinus cinereus
Epilobium angustifolium	Coprinus lagopus
Trifolium sp.	Nil
Reseda luteola	Sterile mycelium of Basidiomycete
Rubus sp.	Coprinus sp.

someone to predict whether the isolated Coprinus species were one of the indigenous flora or just surface propagules on collected vegetation. Nevertheless, the previous chapter has already revealed that there was no isolation of Coprinus species from the detached propagules collected probably by serial washings and inoculated onto agar plates. Whereas the indigenous nature of this species was apparent when growth of these fungi was revealed after the inoculation of washed straw on agar plates. Chang and Hudson (1967) were also unable to find the appearance of any Coprinus species on their dilution plates when they were isolating fungal flora from composted wheat straw.

The criteria used for the isolation of Coprinus species was supposed to be most appropriate, by using aqueous ammonia in the growth media as the presence of ammonia created a highly alkaline condition for the isolation of aklalophilic or ammonia fungi. The term 'ammonia fungi' was used by Sagara (1975) when he isolated a selected group of fungi from uncultivated land after an application of aqueous ammonia. Among his selected isolations, a number of Coprinus species were also listed, e.g. C. echhinosporus, C. neolagopus, C. lagopus, C. narcoticus, C. phylyctidosporus, C. radicosum and C.stercorius. He has included urea fungi and proteophilous fungi by giving them a more general name ammonia fungi, believing that both urea and protein could almost completely be replaced by ammonia when the relative fungi utilized these substances as the sole nitrogen source. However, the work of Fries (1945, 1956) also indicated that the Coprinus species could grow very happily when ammonia is added to the growth medium as the only nitrogen source.

Previous investigators at the Biodeterioration Information Centre (B.I.C.) have also provided the same evidence (Seal, 1973; McShane, 1976; Penn, 1977).

Generally speaking, it is rather difficult to provide further relevant information about the isolation of Coprinus species from vegetation. However, one could draw attention to the early suggestion made by Buller (1933), who indicated that most of the Coprophilous fungi disperse their spores through the agencies of wind, flowering plants and herbivorous animals; the Coprinus species was included in those groups.

CHAPTER 4

Influence of a range of temperatures on the isolation of fungi under alkaline pH conditions.

CHAPTER 4

4.1. INTRODUCTION

This part of the investigation was concerned with studying the influence of a range of temperatures on the isolation of fungi from barley straw under alkaline conditions. Emphasis was made particularly on the isolation of thermotolerant or thermophilic fungi.

For fungal isolation, incubation temperatures ranging from 20°C - 50°C increments of 5°C were used and the isolation techniques employed were similar to the ones described in Chapter 2.

4.1.2. Influence of different incubation temperatures upon the growth and isolation of fungi from straw under alkaline pH conditions

Temperature is one of the major factors influencing the growth rates of fungi. According to a range of their temperature tolerating abilities, they have been classified as phychrophilic, mesophilic and thermophilic (Cooney and Emerson, 1964). It is obvious from previous investigations that fungi as a rule are more tolerant of lower than higher temperatures in comparison with other micro-organisms such as bacteria and yeast. One could consider particularly Basidiomycetes, as the best example of obeying that rule (Humphry and Sigger, 1938) with the exception of *Coprinus* species, which could tolerate-considerably higher degree of temperature in their growth environment, such as *Coprinus fimentarius* (Fries, 1956). A more precise term, thermotolerant, could be used for classifying a large number of fungi, which could exist and develop their growth from 20 - 50°C temperatures. Cooney and Emerson (1964) defined the

thermotolerant fungus as "one that has a maximum temperature tolerant near 50°C and a minimum well below 20°C". These fungi are economically very important for causing bio-deterioration problems. On the other hand, they can also play a vital role in conversion process of economically important agricultural wastes into biologically up-graded feeding products for ruminants. Considering cereal crops in particular, a large number of research investigators have reported the isolation of mesophilic, thermotolerant and thermophilic fungi from those economically important materials. As Flannigan (1969) has reported a large number of mesophilic and thermotolerant fungi can be isolated from dried barley grain by inoculating the grain onto the surface of agar and incubating at 25, 37 and 50°C.

Eastwood (1952) also made an attempt to isolate fungi from two types of composting materials, i.e. from the compost of fresh lawn mowings and from cut barley straw. During the composting process, she recorded the temperature and found a maximum rise to 65°C. She chose 25 and 60°C as the incubation temperatures for the isolation of mesophilic and thermophilic fungi, but apart from Aspergillus fumigatus, she failed to isolate any of the thermotolerant or thermophilic fungi. She reasoned that, at the high temperature, i.e. 65°C, fungi were destroyed at the centre of both kinds of composts. Chang and Hudson (1969) have criticized her isolation technique by saying that Eastwood had selected two extreme temperatures, 25°C and 60°C. The former one was too low for any appreciable growth of thermophilic fungi and the latter too high. They have isolated tentatively a large number of thermotolerant and thermophilic fungi including

mesophilic from wheat straw compost. They have divided their fungi into three different groups. Group one included the mesophilic ones: Alternaria tenuis, Cladosporium herbarium, Aureobasidium pullulan, Aspergillus repens, A. amstelodami, A.versicolor, A.candidus, A.nidulans, Penicillium spp. and the theromphilic Mucor pusillus, and the thermotolerant Abixidiaramosa and A.fumigatus. They placed their other thermophilic isolates in group two, those being Humicola insolens, H.lanuginosa, Chaetomium thermophile, Malbranchea-pulchella var sulfura and Talonyces duponte. Finally, in group three, two thermophilic fungi: Sporotrichum thermophile and Mycelia sterilia were placed with mesophilic Fusarium culmorum, Stysanus stermonitis, Coprinus cinereus, C.megacephalus and Clitopilus pinsilus.

Earlier investigations showed that most of the workers have emphasized the thermophilic fungi as being very active in decomposition of a large number of organic materials. Early work of Miehe (1907) showed the isolation of several thermophilic fungi. He also listed the first thermophilic fungus Mucor pusillis (Lind, 1886), whereas Waksman, Ubreit and Cordon (1939) reported the isolation of Thermomyces sp. when fresh manure was incubated at 50°C. Fergus (1964) has also claimed the isolation of many thermophilic fungi from mushroom compost. Seal and Eggins (1976) have also reported the methods of up-grading of agricultural wastes by thermophilic fungi.

After having considered all the above outlined investigations concerning fungal isolation, an attempt was made in this part of the work to isolate a number of different groups of thermotolerant and thermophilic fungi from barley straw after providing a range of incubation temperatures.

Attention has been paid to isolate the fungi restricted to an alkaline pH condition as maintained in

previous isolation techniques. This could provide information as to whether it would be possible for fungi to isolate at the alkaline pH while incubation temperatures would be variable.

It was noticed by some workers during composting straw that the pH of composting material was mostly found towards neutral to alkaline pH's. One could look at the pH measurement recorded by Eastwood (1952) from her composting material, where she found that during the 5th, 26th, 58th and 90th days of grass composting the pH was recorded as 8.35, 7.64 and 8.6 respectively, with a maximum of 8.64, and after 5 and 20 days composting of barley straw recorded a pH of 7.05 and 7.0 respectively. She was unable to isolate any thermophilic fungi and suggested that during the temperature rise of the composting process the thermophilic bacteria attack the organic nitrogenous materials and liberated ammonia, which causes the rise in pH of composting materials. Furthermore, Chang and Hudson (1967) also recorded the pH of their compost and found that the initial pH of the compost varied between 6 and 7.

4.2. Experimental procedure

Similar fungal isolation methods have been reported here, as already described in chapter 2, for the collection of straw samples, their cutting into equal sizes and inoculation onto various growth media. The media were mineral salt agars with added 1, 0.5, 0.25 and 0.625% aqueous ammonia; glucose-cellulose (GC) and potato dextrose (PD) agars were also prepared according to the methods described previously. Yeast phosphate soluble starch (YS) agar was also prepared under alkaline conditions (see appendix for media preparation)

This agar medium was very selective for the growth and isolation of the thermotolerant and thermophilic fungi, its moisture retaining capacity being due to the presence of starch (Cooney and Emerson, 1964).

4.2.1. Methods used for the isolation of fungi from straw at various incubation temperatures

The following inoculation methods have been used, as outlined below, for the isolation of fungi from straw under alkaline pH conditions and with the influence of various incubation temperatures as: 20, 25, 30, 35, 40, 45 and 50°C.

1: Direct inoculation of straw

The following methods have applied for direct inoculation

a) Inoculation on mineral salt agars with 1, 0.5, 0.25, 0.125 and 0.0625% aqueous ammonia supplemented.

b) Inoculation of straw on GC agar, PD agar and YS agar.

2: Inoculation of washed straw

a) Inoculation on same agars as used in the above outlined section 1:a.

b) Inoculation on same agars as used in the above outlined section 1:b.

3: Inoculation of washings

4: Inoculation of ammonia treated incubated straw

All the above outlined methods have been carried out by the same methods as previously described in Chapter 2. Each set of inoculations were supported by running five replicates and all the sets were incubated for 10 days at 20, 25, 30, 35 40, 45 and 50°C incubation temperatures. After the appearance of fungi on incubated growth media, a similar technique was employed for the subculture, pure culture

studies and identification purposes (see Chapter 2).

4.3. RESULTS

4.3.1. Fungi isolated from the direct inoculation methods a and b

The results of the direct inoculation methods a and b are summarised in Tables 12 a and b for the appearance of different groups of fungi isolated under the influence of a range of incubation temperatures. Aspergillus fumigatus, Chaetomium globosum, Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp. were the most dominant fungal species occurring on all agars and at different ranges of incubation temperatures, i.e. 20, 25, 30, 35, 40, 45 and 50°C. Furthermore, a few more fungi were isolated at varying temperatures using method b where glucose-cellulose (GC), potato dextrose (PD), and yeast phosphate soluble starch (YS) agars were used for the inoculation of straw than with method a, where different percentages of aqueous ammonia (1, 0.5, 0.25, 0.125 and 0.0625%) incorporated mineral salt agars were used for inoculation.

a: Isolation after incubation at 20, 25 and 30°C

By seeing the results provided in Table 12a, one could easily realize that there seemed to be nearly the same groups of fungi isolated at 20, 25 and 30°C. Nevertheless, the most frequent one was Aspergillus fumigatus. Amongst other frequent species were: Chaetomium globosum, Coprinus cinereus, Scopulariopsis brevicaulis, Streptomyces sp. On the contrary, less frequent were found to be Alternaria tenuis, Cephalosporium sp. and Fusarium spp. They were only showing their appearance at lower percentages of ammonia supplemented agars. Furthermore, a few more isolates were also found appearing occasionally from 20-30°C temperature ranges, such as Humicola sp., Scopulariopsis chartarum and Rizopus sp.

Table 12a. Occurrence of fungi, isolated from the direct inoculation of straw pieces on 1(I), 0.5 (2), 0.25 (3), 0.125 (4) and 0.0625 (5)% ammonia-supplemented mineral salt agars, at 20, 25, 30, 35, 40, 45 and 50°C, after 10 days of incubation. (+ = fungus appearance on agar media)

Fungi	20°C					25°C					30°C					35°C					40°C					45°C					50°C				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Alternaria tenuis			+	+	+			+	+	+			+	+	+																				
Alternaria sp										+					+																				
Aspergillus fumigatus	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+		+	+		+	+	+	+	+	+
Aspergillus sp				+	+					+					+																				
Cephalosporium spp					+				+	+				+	+			+	+	+					+				+	+				+	
Chaetomium globosum		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																			
Chaetomium spp				+					+		+	+	+	+	+	+				+					+				+	+					
Coprinus cinereus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+															
Fusarium spp			+	+	+			+	+	+			+	+	+																				
Humicola sp															+				+						+										
Mucor sp															+										+				+						
Paecilomyces sp																				+										+					
Penicillium sp					+					+																									
Scopulariopsis brevicaulis		+	+	+	+		+	+	+	+	+	+	+	+	+			+																	
Scopulariopsis chartarum										+					+																				
Scopulariopsis sp			+										+							+															
Rhizopus sp															+					+															
Streptomyces sp							+	+	+	+	+	+	+	+	+		+		+						+										

Table 12b. Occurence of fungi, isolated from the direct inoculation of straw pieces on glucose-cellulose (GC), Potato dextrose (PD), and yeast phosphate soluble starch (YS) agars, at 20, 25, 30, 35, 40 45 and 50°C, after 10 days of incubation

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Alternaria tenuis	+	+	+	+	+	+	+	+	+	+	+	+									
Alternaria sp	+	+	+	+	+	+	+	+	+												
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus flavus				+			+	+	+		+										
Aspergillus versicolor	+	+	+	+	+		+		+												
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+			+									
Aureobasidium sp				+		+															
Botrytis cineria	+						+														
Cephalosporium spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+
Chaetomium globosum	+	+	+	+	+	+	+	+	+												
Chaetomium spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+			
Cunninghamella elegans							+		+			+									
Coprinus cinereus	+	+		+	+	+	+	+	+	+	+	+									
Cladosporium sp	+		+	+				+													
Fusarium spp	+	+	+	+	+	+	+	+	+	+		+									
Gliocladium roseum	+			+																	
Humicola sp							+			+		+		+		+		+			
Mucor sp	+			+		+				+	+				+						
Myriococcum albomyces									+	+		+	+	+	+		+	+			
Paecilomyces sp	+				+	+	+	+		+		+			+						
Penicillium spp	+	+	+	+'	+	+	+	+	+			+									
Rhizopus sp	+	+	+																		
Scopulariopsis brevicaulis	+	+	+	+	+	+	+	+	+	+	+										
Scopulariopsis chartarum			+		+			+													
Scopulariopsis sp			+	+			+			+											
Screptomyces spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			

b : Table 12b showed the appearance of some more fungal species which have not been seen isolated from part a of the inoculation method. The new species include: Aureobasidium pullulans, Aureobasidium sp., Aspergillus flavus, A.versicolor, Botrytis cineria, Cladosporium sp., Gliocladium sp., Myriococcum albomyces, Paecilomyces sp. and Penicillium spp.

Asperigillus fumigatus, Chaetomium globosum, Coprinus cinereus, Scopulariopsi. brevicaulis, and Streptomyces sp., previously illustrated in table 12a, again showed their frequent appearance on GC, PD and YS agars.

It is important to note that the fungi Alternaria sp., Aspergillus sp., Cephalosporium sp., and Fusarium sp., which were less frequent by the last inoculation method, now show their frequent appearance under these circumstances. This could however justify that these mentioned species were presumably dominant isolates from straw, but were unable to cope with the high ammonia concentration.

Isolation after incubation at 35, 40, 45 and 50°C

a: Table 12a showed that fungus Aspergillus fumigatus was again the most dominant species seen isolated from straw on all above-outlined temperatures and on nearly all the agars used. Moreover, the fungus Coprinus cinereus and Streptomyces sp. were observed at higher incubation temperatures, the former up to 40°C and the latter up to 45°C.

Some other groups of fungi were also seen appearing occasionally at higher temperatures, such as Cephalosporium p(1) Chaetomium sp.(2), Humicola sp.(3), Mucor sp(4), Paecilomyces

sp. (5) and Rhizopus sp (6). Of the six isolates, 1, 2, 4 and 5th were showing their appearance up to 45°C, whereas 3 appeared up to 40°C and 6 up to 35°C.

b: Table 12b showed that the most frequent fungal flora seen isolated from all inoculated agars at 35, 40, 45 and 50°C were Aspergillus fumigatus, Cephalosporium sp., along with Cunninghamella elegans and Myriococcum albomyces, as the former one showed its appearance up to 35°C whereas the latter one was occurring up to 45°C. The rest of the results were found to be the same as reported in Table 12A. The temperature tolerating abilities of Alternaria tenuis and Aspergillus flavus, A. versicolor and Aureobasidium pullulans, were increased on this part of inoculation, as was obvious by seeing their appearance at 35°C incubation.

By reviewing the above outlined results from Tables 12a and b, it could be quite easy to group the isolated fungi into different numbers. In group 1 those fungi could be listed which have shown their appearance from the range 20-50°C incubation temperatures respectively those include: Aspergillus fumigatus and Cephalosporium sp. In the same manner, group 2 would be represented by Chaetomium sp and Humicola sp., Myriococcum albomyces, and Streptomyces sp. which have shown their appearance upto 45°C. Whereas group 3 would include Mucor sp., Paecilomyces sp. and Rhizopis sp., which showed their appearance at 40°C. Finally, group 4 will be represented by Alternaria tenuis, Alternaria sp. Aspergillus flavus, A. versicolor, Aspergillus sp., Aureobasidium pullulans, Fusarium spp and Scopulariopsis brevicaulis.

4.3.2. Fungi isolated from inoculation of washed straw

Results are given in Tables 13 a and b for the isolation of fungi from the washed straw inoculated onto different agars and incubated at a range of temperatures.

The washing techniques depleted a maximum number of surface spores and after inoculation of washed straw on agars, and now it could be assumed that the fungi appearing at different incubation temperatures were indigenous flora from the type of straw used in this investigation. Some fungi isolated by the last (direct inoculation) technique have not been seen at all by the present method. On the contrary, the species of Aspergillus fumigatus was once again found as the most common fungus, appearing on nearly all agars and at all incubation temperatures provided for isolation, i.e. 20, 25, 30, 35, 40, 45 and 50°C respectively.

a : Isolation after incubation at 20, 25 and 30°C

The most dominant fungi isolated at above-outlined temperatures seemed to be Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp., and of course, including Aspergillus fumigatus the most abundant one. These fungi showed their appearance on all agar media to which was added different percentages of aqueous ammonia.

On the contrary, a less abundant appearance of Alternaria tenuis, Alternaria sp., Cephalosporium sp and Fusarium spp. were seen on lower ammonia concentrated agars such as 0.25, 0.125 and 0.0625%. This probably showed their inability either against high ammonia percentages or against highly alkaline conditions for growth. (The same evidence was revealed in Table 12a)

Table 13a Occurrence of fungi, isolated from the inoculation of six successively washed straw pieces on 1(I), 0.5(2), 0.25(3), 0.125(4) and 0.0625(5) ammonia supplemented mineral salt agars, at 20, 25, 30, 35, 40 45 and 50°C after 10 days of incubation

Fungi	20°C					25°C					30°C					35°C					40°C					45°C					50°C				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Alternaria tenuis				+	+				+	+			+	+	+				+																
Alternaria sp				+				+							+																				
Aspergillus fumigatus	+	+		+	+		+	+	+	+	+	+	+	+	+		+	+	+	+		+		+	+		+	+	+	+	+	+	+	+	+
Aspergillus sp			+				+							+				+																	
Cephalosporium spp															+			+							+			+							+
Chaetomium globosum			+		+			+		+		+		+	+																				
Chaetomium sp					+					+					+				+					+											
Coprinus cinereus	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+															
Fusarium spp				+	+			+	+	+				+	+																				
Humicola sp																									+										
Paecilomyces sp																					+				+										
Mucor sp																		+																	
Scopulariopsis brevicaulis		+	+	+	+		+	+	+	+		+	+	+	+																				
Scopulariopsis sp										+				+																					
Myriococcum albomyces																			+																
Streptomyces sp			+		+				+	+		+	+	+	+		+	+	+		+		+		+										

Table 13b Occurrence of fungi, isolated from the inoculation of six successively washed straw pieces, on glucose-cellulose (GC), potato dextrose (PD) and yeast phosphate soluble starch (YS) agars, at 20, 25, 30, 35, 40, 45 and 50°C after 10 days of incubation.

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Alternaria tenuis	+	+	+	+	+	+	+	+	+			+									
Alternaria sp	+		+	+	+	+	+	+	+												
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+	+
Aspergillus sp	+	+	+	+	+	+	+	+	+												
Aureobasidium pullulans	+		+	+	+		+		+												
Cephalosporium spp			+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+	+
Botryotrichum piluliferum							+			+											
Chaetomium globosum	+			+			+		+												
Chaetomium sp	+			+	+		+		+			+			+						
Coprinus cinereus	+	+	+	+	+	+	+	+	+	+	+	+			+						
Fusarium spp	+	+	+	+	+	+	+	+	+		+										
Paecilomyces sp				+							+				+			+			
Scopulariopsis brevicaulis	+	+	+	+	+	+	+	+	+												
Scopulariopsis sp	+	+	+					+				+									
Streptomyces	+	+	+	+	+	+	+	+	+	+	+		+		+						

b : After inoculation of washed straw pieces

After inoculation of washed straw pieces on GC, PD and YS agars, nearly the same results have been obtained by seeing the same groups of previously mentioned fungi as reported on the above outlined section a). However, some new fungal isolates were also seen appearing here, like: Aureobasidium pullulans, Botryotrichum piluliferum and Paecilomyces sp., which were not revealed by the two inoculation methods metniond in part a.

c : Isolation from incubation at 35, 40 45 and 50°C

Nearly the same fungal species have been seen appearing at the above outlined incubation temperatures (see Table 13) in comparison with the fungi reported at above outlined section a. The only difference was the appearance of Rhizopus sp. However, the fungus Humicola sp. was only seen once appearing at 40°C and at 0.0625% aqueous ammonia added agar, whereas Paecilomyces sp and Myriococcum albomyces were found isolated at 35°C on 0.25% and 0.125% ammonia added agars. Fungus Cephalosporium sp again seemed to be a high temperature tolerating species as it was seen appearing at the mentioned temperatures (i.e. 35, 40, 45 and 50°C), although its appearance was restricted at high ammonia percentages. Occasional appearance of Chaetomium sp was also noticeable up to 40°C, whereas Streptomyces sp was seen appearing up to this incubation temperature level. The rest of the dominant fungi which showed their presence were Coprinus cinereus and Aspergillus fumigatus. Nevertheless, the former one was only seen appearing at 35°C; on the contrary, the latter one was still showing its presence at

$50^{\circ}C$ incubation temperature. According to previous literature surveys, the species of C. cinereus could tolerate temperatures up to $45^{\circ}C$ (Fries, 1956); whereas A. fumigatus was listed as thermotolerant (Eastwood, 1952; Cooney and Emerson, 1964; Chang and Hudson, 1969; and Flannigan, 1969)

d: Not a great deal of difference in the results was noticed from the isolation of fungi after inoculation on GC agar, PD agar and YS agar in comparison with the inoculation on ammonia supplemented agars at 35, 40, 45 and $50^{\circ}C$ incubation temperatures. The only difference one could see here was the appearance of Aureobasidium pullulans and Botryotrichum piluliferum at $35^{\circ}C$; while these species were unable to appear at the said temperature by the last inoculation technique (see Part C and Table 12b). On the contrary, no appearance of Myriococcum albomyces was revealed here at any above outlined temperatures.

4.3.3. Isolation from inoculation of washings

The results are summarised in Tables 14a, b, c, d, e and f for the isolation of fungi at various incubation temperatures from the inoculation of washings of straw on GC agar, PD agar and YS agar.

The result obtained by the present technique was very much similar to the one provided in Table 13b for the direct inoculation method. Nevertheless, the only big difference one could notice was the absence of Coprinus cinereus, which was the most dominant fungus isolated by the last two isolation techniques (see sections 4.3.1 and 4.3.2).

The main advantages of this method was based upon the fact that a gradual decrease in the number of fungi was

Table 14a Occurrence of fungi, isolated from the inoculation of 1st washing of straw, on glucose-cellulose (GC), potato dextrose (PD) and yeast phosphate soluble starch (YS) agars at 20, 25, 30, 35, 40, 45 and 50°C, after 10 days of incubation. (+ = indicates the appearance of fungi on agar media.)

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Alternaria tenuis	+	+	+	+	+	+	+	+	+												
Alternaria sp	+	+	+	+	+	+	+	+	+			+									
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus spp	+	+	+	+	+	+	+	+	+		+										
Aspergillus flavus	+		+	+		+	+	+													
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+	+											
Aureobasidium sp	+	+	+	+	+	+	+	+	+		+										
Botrytis cineria						+	+		+												
Cephalosporium spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+
Chaetomium globosum	+	+	+	+	+	+	+	+	+												
Chaetomium spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					
Cunninghamella sp							+				+										
Cladosporium sp				+			+		+												
Fusarium spp	+	+	+	+	+	+	+	+	+			+									
Gliocladium roseum	+		+				+														
Humicola sp										+		+			+	+		+			
Mucor sp							+		+	+	+	+	+	+			+	+			
Paecilomyces sp				+	+		+	+	+	+	+	+	+	+	+						
Penicillium spp	+	+	+	+	+	+	+	+	+		+										
Rhizopus sp						+	+	+				+									
Scopulariopsis brevicaulis	+	+	+	+	+	+	+	+	+	+	+										
Scopulariopsis sp	+	+	+	+	+	+	+	+	+	+											
Streptomyces sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						

Table 14b Occurence of fungi isolated from the inoculation of 2nd washing of straw, on glucose-cellulose (GC), potato dextrose (PD) and yeast phosphate soluble starch (YS) agars, at 20, 25, 30, 35, 40, 45 and 50°C. after 10 days of incubation.

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Alternaria tenuis	+	+	+	+	+	+	+	+	+												
Alternaria sp	+	+	+	+	+	+	+	+	+	+											
Aspergillus fumigatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus spp	+	+	+	+	+	+	+	+	+	+	+										
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+			+									
Aureobasidium sp	+	+	+	+	+	+	+	+	+	+											
Botrytis cineria			+			+		+													
Cephalosporium spp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Chaetomium globosum	+	+	+	+	+	+	+	+	+	+											
Chaetomium sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+				
Fusarium spp	+	+	+	+	+	+	+	+	+		+										
Mucor sp											+			+							
Paecilomyces sp					+							+		+	+						
Penicillium spp	+	+	+	+	+	+	+	+	+	+											
Rhizopus sp							+					+			+						
Scopulariopsis brevicaulis	+	+	+	+	+	+	+	+	+	+	+										
Scopulariopsis sp	+		+	+	+		+	+	+												
Streptomyces sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						

+ indicates the appearance of fungus on agar plate

Table 14c Occi̇rence of fungi, isolated from the inoculation of 3rd washing of straw, on glucose-cellulose (GC), potato dextrose (PD), and yeast phosphate soluble starch (YS) agars at 20, 25, 30, 35, 40, 45 and 50°C after 10 days of incubation

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Alternaria tenuis	+	+	+	+	+	+	+	+	+												
Alternaria sp		+			+		+	+													
Aspergillus fumigatus		+			+	+	+	+	+		+	+		+	+		+	+		+	
Aspergillus sp	+			+	+	+	+	+	+												
Cephalosporium sp					+		+	+	+		+		+		+		+				+
Chaetomium globosum	+	+	+	+	+	+	+	+													
Chaetomium sp				+	+	+		+	+			+			+						
Fusarium sp	+	+	+	+	+			+													
Paecilomyces sp												+									
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+												
Penicillium spp	+	+	+	+	+	+	+	+	+												
Scopulariopsis brevicaulis	+	+	+	+			+	+	+			+									
Scopulariopsis chartarum								+													
Scopulariopsis sp			+					+	+												
Streptomyces sp	+	+	+	+	+	+	+	+	+		+			+							

+ = indicates the appearance of fungus on agar plate

Table 14d Occurence of fungi, isolated from the inoculation of 4th washing of straw, on glucose-cellulose (GC), potato dextrose (PD) and yeast phosphate soluble starch (YS) agars, at 20, 25, 30, 35, 40, 45 and 50°, after 10 days of incubation

Fungi	$20^{o}C$			$25^{o}C$			$30^{o}C$			$35^{o}C$			$40^{o}C$			$45^{o}C$			$50^{o}C$		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Aspergillus fumigatus		+		+	+		+	+	+			+	+	+			+				+
Aspergillus sp	+		+		+				+	+											
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+	+											
Aureobasidium sp		+	+					+													
Botrytis sp								+													
Chaetomium globosum			+			+		+	+												
Chaetomium sp							+														
Penicillium sp	+	+	+	+	+	+	+	+	+												
Scopulariopsis brevi-caulis					+			+	+			+									
Scopulariopsis sp						+			+												
Streptomyces sp					+		+		+			+			+						

+ indicates the appearance of fungus on agar plate

Table 14e Occurence of fungi, isolate from the inoculation of 5th washing of straw, on glucose-cellulose (GC), potato dextrose (PD), and yeast phosphate soluble starch (YS) agars at 20, 25, 30, 35, 40, 45 and 50°C, after 10 days of incubation

Fungi	20°C			25°C			30°C			35°C			40°C			45°C			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Aspergillus fumigatus		+		+			+		+	+		+	+				+				
Aspergillus sp	+			+			+		+												
Auréobasidium pullulans	+	+	+	+		+		+	+												
Penicillium sp	+	+	+	+		+			+												

+ = indicates the appearance of fungus on agar plate

Table 14f Occurrence of fungi, isolated from the inoculation of 6th washing of straw, on glucose-cellulose (GC), potato-dextrose (PD) and yeast phosphate soluble starch (YS) agars, at 20, 25, 30, 35, 40, 45 and 50°C after 10 days of incubation

Fungi	20°C			25°C			30°C			35°C			40°C			45°			50°C		
	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS	GC	PD	YS
Aspergillus fumigatus							+							+							
Aspergillus sp			+		+			+													
Aureobasidium pullulans	+		+	+		+	+		+												
Penicillium sp	+		+						+												
Streptomyces sp									+			+									

+ indicates the appearance of fungus on agar plate

noticed with 1-6 successive washings from straw being inoculated on agars and incubated at 20, 25, 30, 35, 40, 45 and 50°C. This has provided a more clearcut picture of the isolation and identification criteria for the sporal propagules which were detached from straw, while the direct inoculation methods have caused the overlapping of most of the fast growing fungi after intermingling their growing mycelia on agar plates.

Isolation at 20, 25 and 30°C from first washing

The most dominant fungi found isolated on GC agar, PD agar and YS agar were *Alternaria tenuis*, *Alternaria* sp. *Aspergillus fumigates*, *A. flavus*, *Aspergillus* sp., *Aureobasidium pullulans*, *Chaetomium globosum*, *Chaetomium* spp *Fusarium* spp., *Penicillium* spp., *Scopulariopsis brevicaulis*, *Scopulariopsis* sp. and *Streptomyces* sp., whereas the occasional appearances of *Botrytis cineria*, *Cunninghamella* sp., *Cladosporium* sp., *Gliocladium roseum*, *Paecilomyces* sp. and *Rhizopus* sp. were noticed. These fungi were also ragarded as atmospheric contaminants; their presence on straw and stored grain was reported previously by many investigators (Tuite and Christensen, 1955; Flannigan, 1969).

Isolation from second washing

Although nearly the same groups of fungi were found isolated here by the same ratio as was noticed by the inoculation of the first washing on agars, the number of isolates seemed to be in decreasing order.

Isolation from third washing

A further decrease in the number of detached propagules from straw was revealed on the inoculated agars from the third washing. In addition to that, depletion of *Aspergillus flavus*, *Botrytis cineria*, *Cunninghamella* sp., *Cladosporium* sp., *Humicola* sp., *Mucor* sp. and *Rhizopus* sp. was noticed. This could probably be due to the complete detachment of their spores during first and second washing processes of straw. However, the rest of the isolated fungi still remained the same in the range of similar order of appearance, as was revealed before from the first washing inoculation.

Isolation from fourth washing

With the gradual decrease in the number of fungal propagules the depletion of dominant species was noticed here from the absence of *Alternaria tenuis*, *Alternaria* sp., *Cephalosporium* sp., *Fusarium* sp and *Paecilomyces* sp.

Isolation from fifth washing

A far less number of fungi were seen appearing by the inoculation of the fifth washing on agars. Isolates appearing include: *Aspergillus fumigatus*, *Aspergillus* sp., *Aureobasidium pullulans* and *Penicillium* spp.

Isolation from sixth washing

Some species of fungi reported from the fifth washing were seen appearing occasionally with the addition of *Streptomyces* sp. This showed the surface retaining abilities of some of their spores even after the fifth and sixth washings of the straw. There could be further chances of their appearance even after the inoculation of further washings on agars, but no attempt was made beyond this level.

4.3.4. Isolation from ammonia amended, inoculated straw at different incubation temperatures

Table 15 showed the results provided for the effect of different temperatures upon the isolation of fungi from ammonia treated straw, inoculated on to glucose-cellulose agar.

The reason for selecting this technique was to provide further chances for the isolation of those fungi which were unable to appear from straw pieces plated out directly onto agars which had been incorporated with different precentages of aqueous ammonia (see Table 13a). Nevertheless, they revealed their appearance when direct inoculation of straw was made on GC agar, PD agar and YS agar. Now the results seemed to be quite similar to the ones listed in Table 13b. This showed the abilities of most of the fungi to tolerate high percentages of ammonia while they were exposed on those environments. These fungi include: *Aspergillus flavus*, *Aureobasidium pullulans* and *Aureobasidium* sp.

Isolation at 20, 25 and 30°C

Alternaria tenuis, *Alternaria* sp., *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Aureobasidium* sp., *Coprinus cinereus*, *Fusarium* spp and *Scopulariopsis brevicaulis* were found as the most dominant fungal flora isolated after incubation at the above-outlined temperatures. While the appearance of *Botryotrichum piluliferum* and *Stachybotrys atra* was only revealed once from 30°C incubated petridishes.

Isolation at 35, 40, 45 and 50°C

The most dominant species seen were *Aspergillus fumigatus*, *Cephalosporium* sp., *Paecilomyces* sp and *Mucor*. The first and

Table 15. Effect of different incubation temperatures upon the isolation of fungi from straw pieces (which had previously been amended with 1, 0.5, 0.25, 0.125 and 0.0625% ammonia cellulose agar for 10 days incubation

Fungi	$20^{o}C$	$25^{o}C$	$30^{o}C$	$35^{o}C$	$40^{o}C$	$45^{o}C$	$50^{o}C$
Alternaria tenuis	+	+	+	+			
Alternaria sp	+	+	+	+			
Aspergillus fumigatus	+	+	+	+	+	+	+
Aspergillus flavus	+	+	+				
Aspergillus sp	+	+	+	+			
Aureobasidium pullulans	+	+	+	+			
Aureobasidium sp		+	+				
Botryotrichum piluliferum			+	+			
Cephlosporium spp	+	+	+	+	+	+	
Chaetomium globosum	+	+	+				
Chaetomium spp	+	+	+	+	+		
Coprinus cinereus	+	+	+	+			
Fusarium spp	+	+	+	+			
Myriococcum albomyces				+	+		
Paecilymoces sp				+	+	+	
Mucor sp.				+		+	+
Scopulariopsis brevicaulis	+	+	+	+			
Scopulariopsis chartarum			+				
Scopulariopsis sp		+	+				
Stachybotrytis atra			+				
Streptomyces sp	+	+	+	+	+		

+ indicates the appearance of fungus on agar plate.

fourth showed their appearance even at 50°C incubation temperature, while the second and third ones appeared up to 45°C. The species of Chaetomium sp and Streptomyces sp. were able to appear up to 40°C, while less temperature tolerant were found to be Alternaria tenuis, Alternaria sp, Aureobasidium pullulans, Aspergillus sp., Botryotrichum piluliferum. coprinus cinereus, Fusarium sp., which appeared only up to 35°C.

4.3.5. Thermophilic activities of isolated fungi by pure culture inoculation

All the fungi isolated from the present isolation programme were tested for their thermophilic activities in pure cultures. This was achieved by inoculating the pure cultures on yeast phosphate soluble starch agar and incubated at 30, 35, 40, 45 and 50°C. The results are provided in Table 16 by taking the average of four replicates for each fungus.

The table showed that the fungi Aspergillus fumigatus, Cephalosporium sp. and Humicola sp. were showing their growth at all the above-outlined incubation temperatures, while Botryotrichum piluliferum, Chaetomium sp., Myriococcum albomyces, Paecilomyces sp. and Streptomyces sp. revealed their growth up to 45°C, whereas Coprinus cinereus, Cunnihghamella elegans and Rhizopus sp. were able to tolerate 40°C, showing their mycelial growth up to this temperature. The rest of the fungi could only cope up to 35°C excluding Arthobotrytis sp. Alternaria sp., Aspergillus sp. Botrytis cineria, Cladosporium sp., Glicocladium roseum, Penicillium spp. Scopulariopsis sp and S. chartarum.

Table 16. Effect of high incubation temperatures on the growth of pure cultures of isolated fungi, inoculated onto yeast phosphate soluble starch agar and incubated for 10 days at 30, 35, 40, 45 and 50°C.

Fungi	30°C	35°C	40°C	45°C	50°C
Arthbotrytis sp	+				
Alternaria tenuis	+	+			
Alternaria sp	+				
Aspergillus fumigatus	+	+	+	+	+
Aspergillus flavus	+				
Aspergillus versicolor	+	+			
Aspergillus niger	+	+			
Aspergillus spp	+				
Aureobasidium pullulans	+	+			
Botrytis cineria	+				
Botryotrichum piluliferum	+	+	+	+	
Cephalosporium spp	+	+	+	+	+
Chaetomium globosum	+	+			
Chaetomium spp	+	+	+	+	
Cladosporium sp	+				
Coprinus cinereus	+	+	+		
Cunninghamella elegans	+	+	+		
Fasarium spp	+	+			
Gliocladium roseum	+	+			
Humicola sp	+	+	+	+	+
Mucor sp	+	+	+	+	
Myriococcum albomyces	+	+	+	+	
Paecilomyces sp	+	+	+	+	
Penicillium spp	+				
Rhizopus sp	+	+	+		
Scopulariopsis brevicaulis	+	+			
Scopulariopsis chartarum	+				
Scopulariopsis sp	+				
Stachybotrys atra	+	+			
Streptomyces sp	+	+	+	+	

+ indicated the appearance of fungus on agar plate

4.3.6. Measurement of colony diameter of some selected fungi after incubation at higher temperatures

Twelve isolated fungi were selected from the preliminary examinations reported in Section 4.3.4. for the effect of higher incubation temperatures upon isolated fungi. However, these twelve fungi were further inoculated by cutting 6 mm growing mycelial plugs from the edges of potato dextrose agar (as stock cultures were maintained on this agar) and placing them at the centre of YS agar. Incubation was made for one week at 30, 35, 40, 45 and 50°C respectively, for measuring the colony diameter after growth with the influence of a range of higher temperatures.

Table 17 and Figure 1 provided the results for their colony diameter and each figure represents the averages from 4 replicates for an individual fungus. Figure 1 showed that at 50°C incubation temperature, the highest colony diameter was recorded for Humicola sp as 5.00 cm. Most of the fungi including Aspergillus fumigatus, Chaetomium sp., Mucor sp., Rhizopus sp. and Coprinus cinereus showed their maximum growth upto 35°C, while their growth degreased gradually with the further increase in temperature, whereas Cephalosporium sp., Humicola sp. and Myriococcum albomyces showed their maximum at 35°C. On the contrary, Botryotrichum piluliferum, Paecilomyces sp. and Streptomyces sp showed their maximum colony diameters at 30°C.

Table : I7 Measurement of colony diameter of selected fungi after incubation at higher temperatures for ten days.

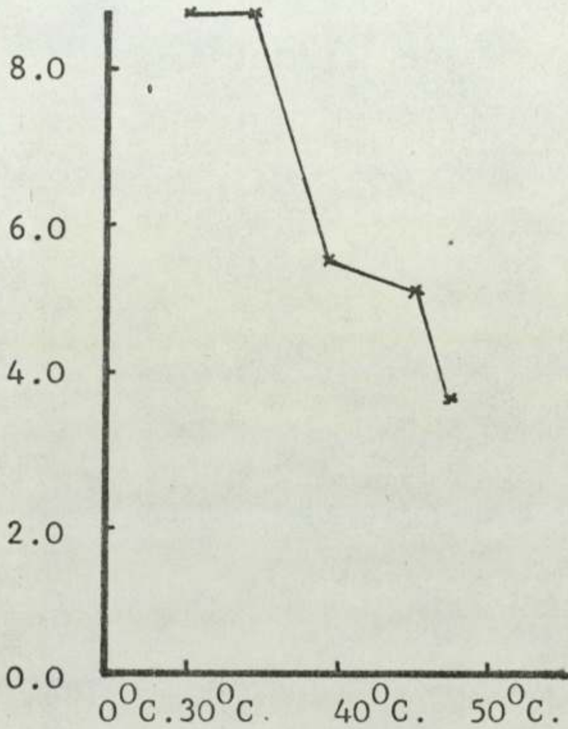
FUNGI	Measurement of colony diameter in centimetres after incubation at different temperatures.				
	30°C.	35°C.	40°C.	45°C.	50°C.
Aspergillus fumigatus	8.50	8.50	5.50	4.80	3.50
Botryotrichum piluliferum	8.50	7.8o	4.50	–	–
Cephalosporium sp.	6.00	8.50	7.50	6.50	3.50
Chaetomiumsp	8.50	8.50	7.20	2.30	–
Gliocladium roseum	8.00	7.00	2.50	–	–
Humicola sp.	5.50	8.50	8.50	7.80	–
Mucor sp.	8.50	8.50	4.50	3.30	–
Myriococcum albomyces	6.50	7.50	8.50	4.80	–
Paecilomyces sp.	6.80	3.00	2.20	I.90	–
Rhizopus sp.	8.50	8.50	2.I0	–	–
Coprinus cinereus	8.50	8.50	4.00	–	–
Streptomyces sp.	4.00	3.I0	2.00	0.50	–

– = Indicates no growth.

Figure: 1

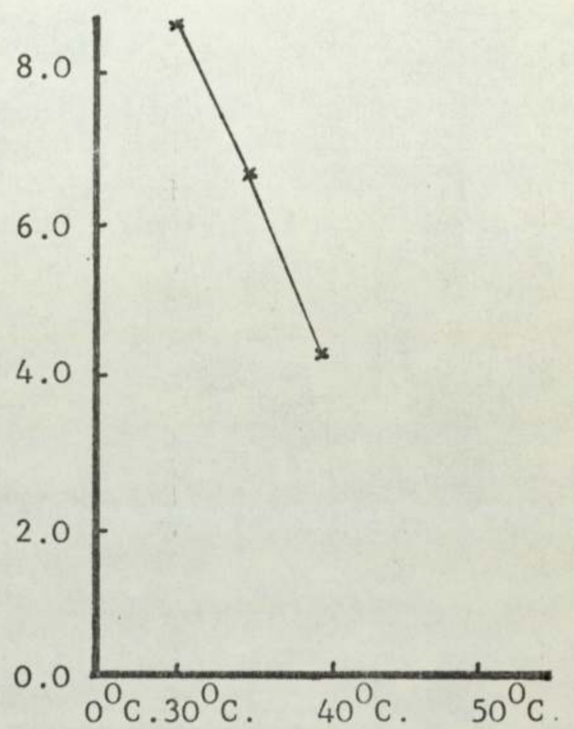
Fungal colony diameter at different incubation temperatures

Mycelial diameter (cm.)

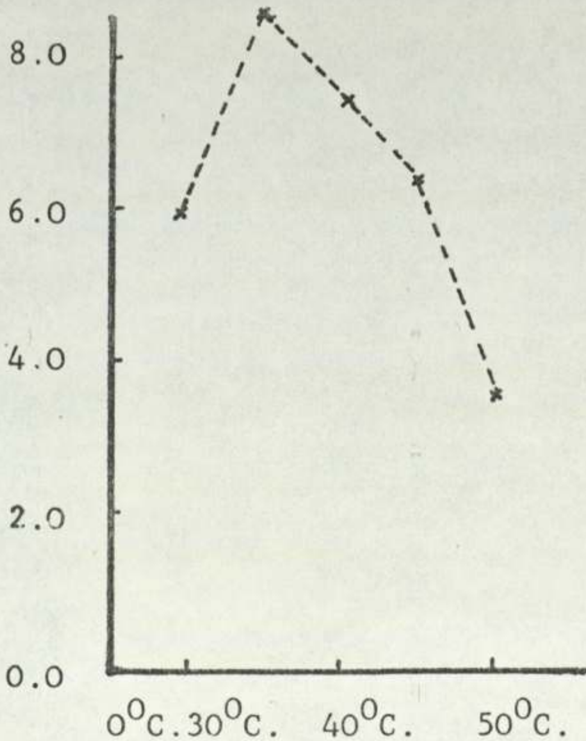


Aspergillus fumigatus

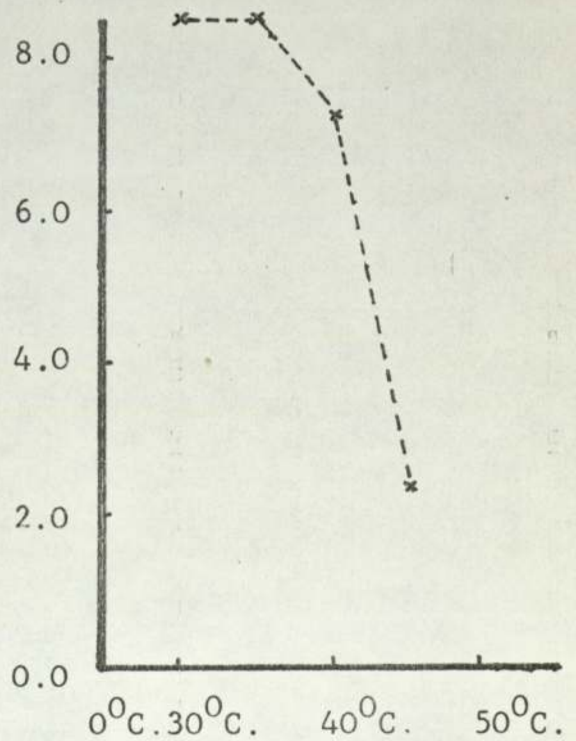
Mycelial diameter (cm.)



Botryotrichum piluliferum

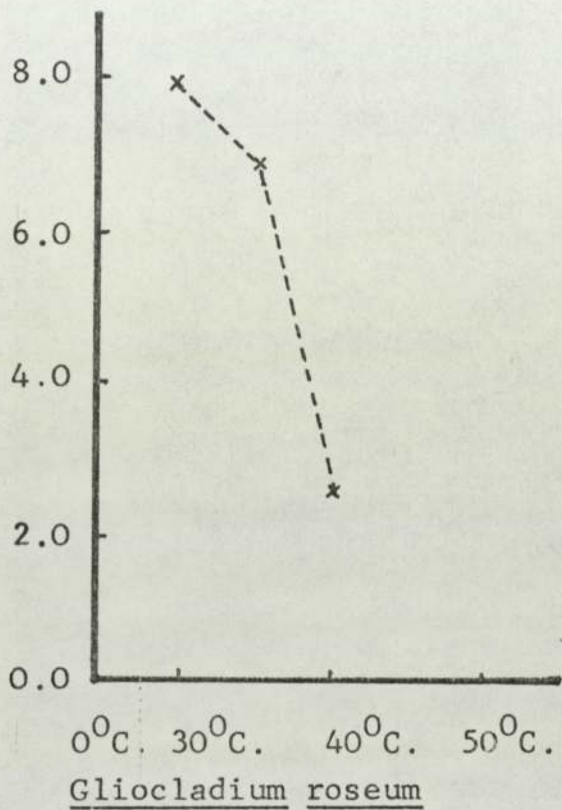


Cephalosporium sp

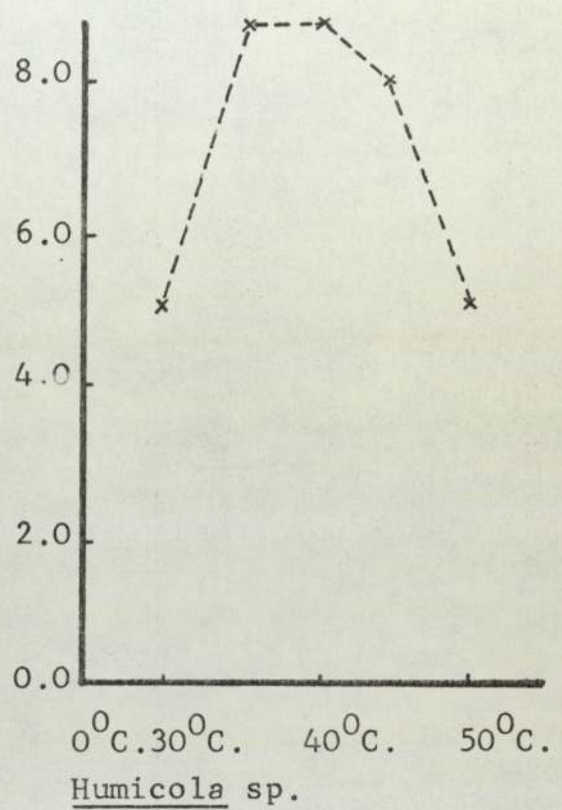


Chaetomium sp

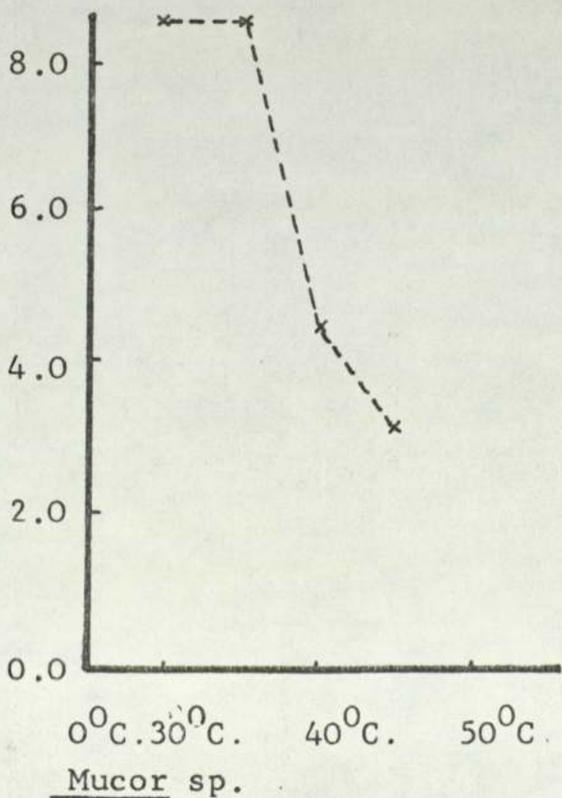
Figure 1 (cont.)



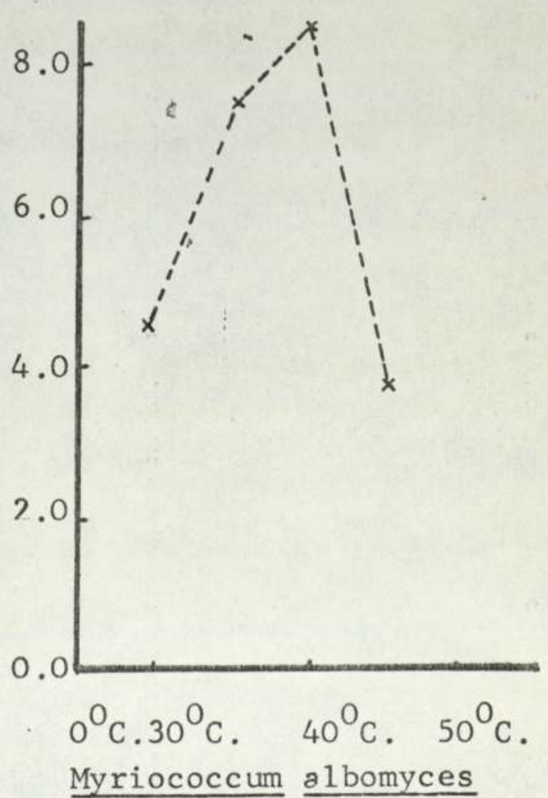
Gliocladium roseum



Humicola sp.

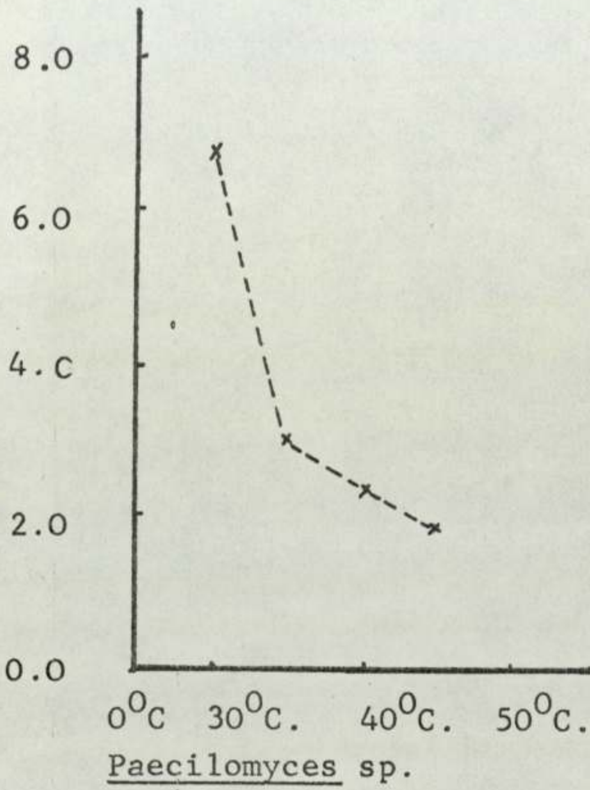


Mucor sp.

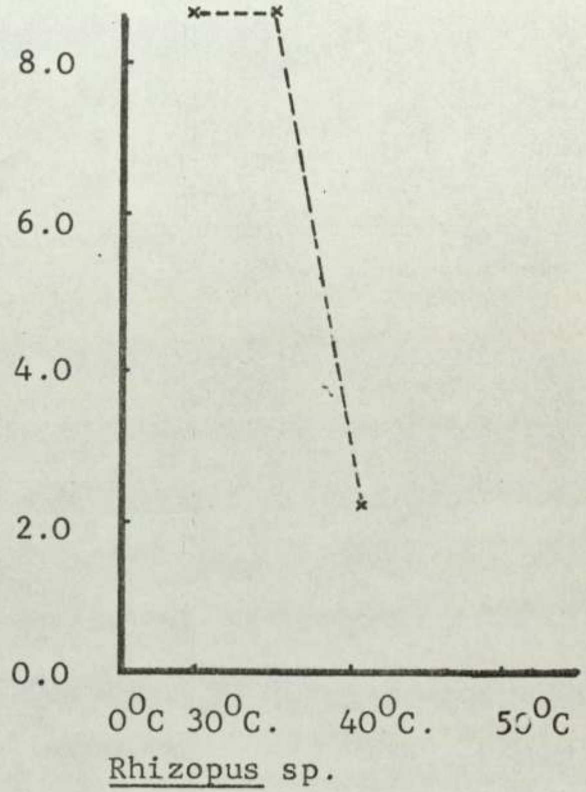


Myriococcum albomyces

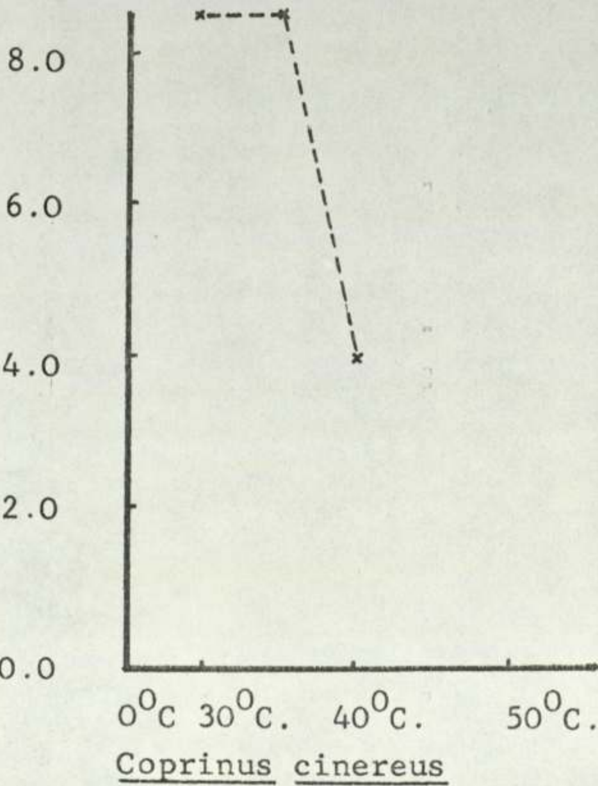
Figure 1. (Cont.)



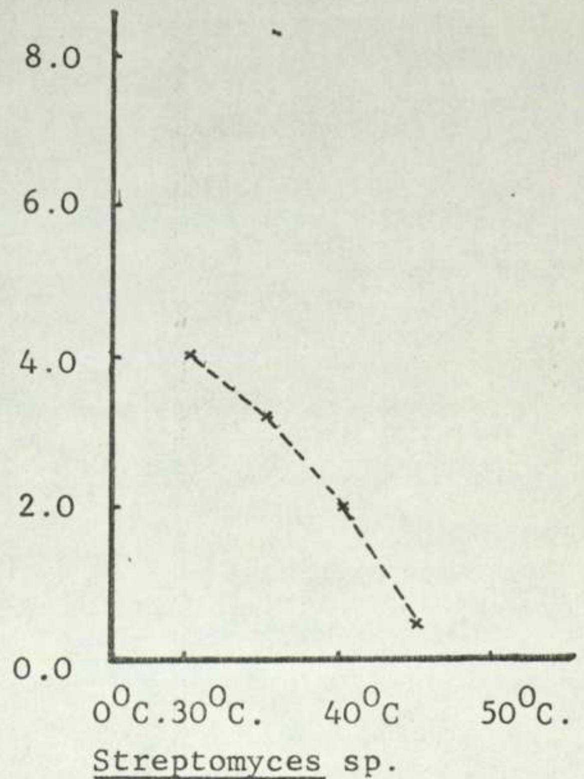
Paecilomyces sp.



Rhizopus sp.



Coprinus cinereus



Streptomyces sp.

4.4. DISCUSSION

The results of the present work showed the isolation of fungi from straw under the influence of a range of incubation temperatures and under alkaline pH.

After having applied various isolation techniques, a number of mesophilic and thermotolerant (Cooney and Emerson, 1964) fungi have been seen appearing on agars during incubation at 20, 25, 30, 35, 40, 45 and 50°C temperatures. During incubation at these temperatures, the most common fungi seen appearing were *Aspergillus fumigatus* and *Cephalosporium* sp., although *Mucor* sp. was also seen occurring at the mentioned temperatures, but its appearance was occasional, mostly depending upon the technique suitable for its isolation. Moreover, *Humicola* sp. was also noticed occasionally on agar media up to 50°C, but its isolation was not revealed at lower temperatures. Occasional appearances of *Botryotrichum piluliferum* and *Cunninghamella elegans* were also reported from 30-35°C, but they were unable to appear below the stated temperature ranges. On the contrary, *Paecilomyces* sp. and *Rhizopus* were found occurring up to 20°C, and it was noticed that the former was unable to establish its appearance above 35°C. An abundant appearance of *Coprinus cinereus* and *Streptomyces* sp. were revealed from 20-35°C respectively, but the latter one was still seen appearing occasionally at 40°C as well.

Most of the mesophilic fungi showed their appearance up to 35°C, but they were less abundant at that temperature, in comparison with their growth at lower degrees of incubation temperature, i.e. 20, 25 and 30°C where their frequencies of occurrence were higher. Among those groups the most common ones were *Alternaria tenuis*, *Alternaria* sp.,

Aspergillus sp., *Aureobasidium pullulans*, *Chaetomium globosum*, *Fusarium* spp., *Scopulariopsis brevicaulis* and *Scopulariopsis* sp., with the occasional isolation of *Aspergillus flavus*, *A. versicolor*, *A. niger*, *Botrytis cineria*, *Cladosporium* sp., *Gliocladium* sp., *Penicillium* spp and *Scopulariopsis chartarum*.

Twelve isolated fungi were selected for their growth measurement at higher incubation temperatures. It was found that at the range of 30-50°C, most of the fungi have shown their maximum colony diameters such as *Aspergillus fumigatus*, *Botryotichum piluliferum*, *Cephalosporium* sp., *Chaetomium* sp., *Coprinus cinereus*, *Cunninghamella elegans*, *Mucor* sp., *Paecilomyces* sp., *Rhizopus* sp. and *Streptomyces* sp. Whereas, *Humicola* sp. showed maximum growth at 40-45°C, on the other hand, the fungus *Myriococcum albomyces* only revealed its maximum mycelial growth at the former temperature.

CHAPTER 5

Utilization of various carbon sources by the isolated fungi

Chapter 5

5.1. Introduction

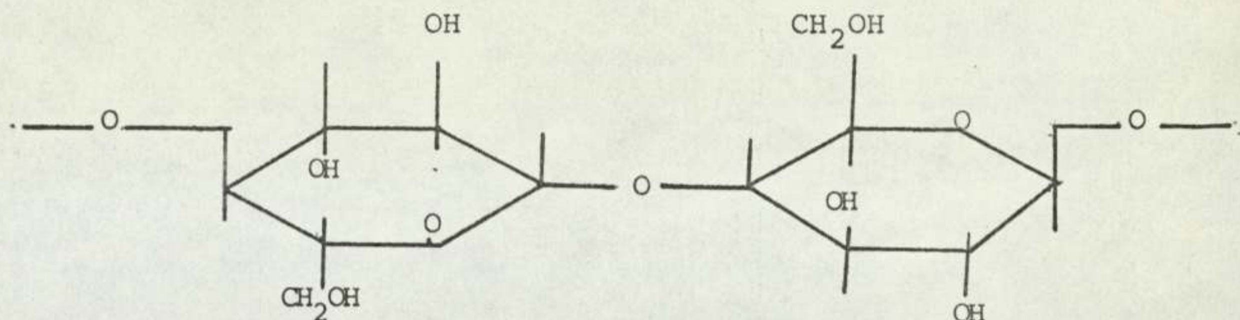
After isolation of fungi from barley straw under alkaline conditions the utilization of various carbon sources was investigated. The identified fungi were inoculated onto agar media incorporating cellulose, hemicellulose, ball milled straw and lignins, respectively, as the sole carbon sources. As one of the aims of the investigation was to select fungi for the biological up-grading of barley straw, the criteria of selection were based on the lignocellulolytic characteristics.

A preliminary screening experiment was conducted on the isolates to test their relative abilities to utilise the various carbon sources. Each isolate was inoculated onto media containing individual carbon sources and their relative abilities to utilise each carbon source determined by the growth rates and by cleaning of the media of all carbon sources - except lignin, where apart from growth measurement from liquid and solid media (containing lignins as the sole carbon sources), a Bavendamms Polyphenoloxidase test was also performed to test their ligninolytic activities.

5.1.2. Utilization of various carbon sources by fungi

5.1.2.1. Cellulose:

Cellulose is the basic unit of the structural framework of the plant. It forms the major portion of the plant cell walls. The cellulose molecule is formed by linking D-Glucose residues through B1-4 linkage, forming large chains. Its empirical formula is ($C_6H_{10}O_5$) and the structural formula is given below:



woody plants are composed of over 50% cellulose and barley straw contains over 40% of this material (Thomas, 1956; Peitersen, 1975). In nature, cellulose molecules are aggregated into microfibrills, each with a width of 50-200°A. Highly ordered regions of microfibrills are called crystallites or micelles, which are the purest form of cellulose. More random ordered regions are called amorphous or paracrystalline (Ranby and Rhdalm, 1956; Preston and Cronshaw, 1958; Cowling, 1959; Rollins and Tripp, 1961; Cowling, 1963; Muhthehaler, 1967; Cowling and Brown, 1969; Ranby, 1969; Isherwood, 1970 and Hesset et al, 1975).

In nature, cotton seed hairs are called 'native' cellulose (the purest form of cellulose) with more than 90% cellulose and minor amounts of waxes and other materials. In this type of cellulose there is a high proportion of resistance crystalline regions which are only capable of being degraded by the cellulases of certain micro-organisms.

5.1.2.2. Fungal degradation of Cellulose

Among different classes of fungi, the ascomycetes and the fungi imperfecti play an important role as plant residue decomposer in soil (Norkrans, 1963); and detailed studies have been carried out by many workers to evaluate the

breakdown processes by these fungi (Waksman and Skinner, 1926; Siu, 1951; Cascoigne, 1960; Garrett, 1963; Chang, 1969; Domsch and Gams, 1969, Fergus, 1969).

5.1.2.3. Mechanism of cellulose Degradation

It is believed that the enzymes are responsible for the breakdown of cellulosic substrates. These enzymes are collectively known as cellulases, which are synthesised by the cellulolytic microorganisms. The mechanisms of Cellulose attack are dependant upon its three components, viz: I) C_1, II) Cx and III) the β-glucosididoses. Hitherto, it is presumed that the C_1 component is capable of separating the crystalline regions of cellulose. The Cx component attacks the more amorphous regions of the chain. β-glucosidases is capable of hydrolyzing the smaller oligomers. (Siu, 1951).

5.1.3. Lignin

Lignin is an essential component of the supporting tissue of plants and is found in close association with cellulose. The term lignin is derived from the Latin word 'lignum', meaning wood and is defined as a polymeric natural product arising from an enzyme initiated dehydrogenative polymerization of three primary precursor alchols: I) trans-coniferyl, II) trans-synapyl and III) p-coumaryl-alcohols (Sarkanen and Ludwig, 1971).

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5.1.3.1. Lignin Degradation

Due to its complex nature and structure, lignin acts as a barrier against microbial attack; however, it has been shown that certain fungi, particularly the Basidiomycetes, play an important role in the breakdown of lignified plant materials. The Basidomycetes are wood-rotting fungi known as the white and the brown-rot fungi after an investigation made by Bavendamm (1928). His test was based upon the production of an extracellular enzyme,polyphenoloxidase,on phenol containing medium (gallic or tannic acid, etc). He considered the test as positive after the appearance of dark brown diffusion zones on agar medium,and the fungi responsible for this reaction werecalled the white-rots, and the agents for lignin decomposition. On the contrary, fungi which failed to show the above reaction wereknown as the brown rots and are not considered as the lignin degraders. Several other investigators have substantiated Bavendamm's reactions with some modifications and have come to similar conclusions. (Davidson, Campbel and Blaisdell, 1938; Preston, 1948; Law, 1950; Pew, 1951; Dion, 1952; Etheridge, 1957; Kirk and Kelman, 1965.)

5.1.4. Hemicellulose

Hemicellulose is the carbohydrate that, next to cellulose, occurs most abundantly in nature, particularly associated with the plant cell wall and normally designated as xylan, which is generally not soluble in cold water but in hot water and diluted alkali (Sorensen, 1957).

The hemicelluloses present in straw are mainly pentosans,

which, according to Chang (1967), consisted of about 35% of the dry weight of straw. This group of polysacchaside is also sensitive against a wide range of micro-organisms which could, however cause degradation of its components. Schmidt, Petersen and Fr (1923) reported the decomposition of 35-50% of the pentosans in corn forage and oat straw within 100-300 days, while the genera of the soil fungi such as Aspergillus, Cunninghamella and Rhizopus were inoculated with these substrates. Several other investigators have also reported tentatively the active roles of mixed microbial population and particularly fungi towards hemicelluloses decomposing abilities (Tenny and Waskman, 1929 Norman, 1939; Waksman, 1939; Domsch and Gams, 1969 and Flannigan, 1970, 1972).

5.1.5. A New Enzyme System for Lignocellulose Degradation

Westermark and Eriksson (1974 a and b) discovered a new extracellular enzyme , Cellobiose quinone-oxidoreductase, while growing a white-rot fungus polyporus versicolor on liqnin agar plates supplemented with cellulose. They suggested that this enzyme was formed due to the presence of cosubstrate-cellobiose - a degradation product of cellulose, causing the reduction of quinones and phenoloxyradicals which results in lignin degradation. Further studies made by Hiroi, Eriksson and Stenlund (1976 a and b) have indicated the growth of a white-rot fungus Pleurotus ostreatus, in lignosulphonate and lignosulphonate and cellulose supplemented growth medium. A decrease in phenolic hydroxylic content was estimated in both types of medium.

5.2. EXPERIMENTAL PROCEDURE

5.2.1. Preparation of stock cultures of isolated fungi

Isolated fungi were maintained on potato dextrose agar, adjusted at pH 7.7 using Sorensens Phosphate buffer solutions (Lindeberg, 1939, 1944) and subcultured at monthly intervals. Most of the fungi were kept at 30°C incubation temperature, while some thermotolerant species, such as *Chaetomium* sp. *Humicola* sp and *Myriococcum albomyces*, were incubated at 35°C and 40°C as well.

5.2.2. Preparation of culture inoculum

Culture inocula were prepared by inoculating original stock cultures in malt extract broth placed in 100 ml conical flasks with 20 ml aliquots. These flasks were incubated for one week by transferring them to an orbital shake-table in a large incubator maintained at 30°C. The mycelial pellets which formed were removed aseptically from the liquid media and washed thoroughly with sterile distilled water and then suspended in buffered sterile water. (Day, Pelczar and Gottlieb, 1949). These stock pellets were used as inocula for further inoculation programmes.

5.2.3. Substrates used

The following substrates were used as the sole carbon sources in the growth media detailed below:

a. 1% cellulose suspension, prepared by ball-milling for 72 hours to reduce the size of the particles.

b. Xylan, produced by Sigma London Chemical Co. Ltd.

c. Lignin-Indulin A(R) (source: Polychemical Division,

Polychemical Department, North Charleston, South Carolina, 29406, U.S.A.)

d. Lignosulphonate - (a produce from George-Pacific Corporation, Bellingham Division, Bellingham, Washington, U.S.A)

e. Lignin - prepared from barley straw by 72% H_2SO_4 method (see appendix)

f. Ball-milled straw suspension: ground straw was ball-milled for 72 hours to obtain homogeneous suspension.

5.2.4. Preparation of growth media

i) Eggins and Pugh Cellulose agar:

Eggins and Pugh's cellulose agar was prepared (see appendix) and pH adjusted at pH 7.7 using Sorensen Phosphate buffer solutions (Lindbergs, 1939, 1944).

ii) Basal Media:

Without any addition of carbon sources, basal media were prepared by weighing out 1 gm kH_2PO_4, 0.5g$(NH_2)_2SO_4$, 0.5 g Kcl, 0.2gm MgSo4 7 H_2O, 0.1 gm $CaCl_2$, 15 gms agar and distilled water to 1 litre. For liquid media preparation the components were the same as those used for solid media, except for the omission of agar.

iii) Gallic and Tannic Acid (0.5% each) agar:

Previously autoclaved malt agar (2.5% malt extract, 1.5% agar agar) was incorporated separately into 0.5% gallic and the tannic acids and pH obtained were found to be 5.6 and 5.5 respectively for both types of agars. The phenolic acids were dissolved previously in measured quantities of hot sterile distilled water.

5.2.5. Inoculation of isolated fungi on media supplemented with different carbon sources

a) Inoculation on Eggins and Pugh Cellulose agar

The molten cellulose agar (Eggins and Pugh, 1962) was cooled to 45°C by keeping the agar flask in a bath of hot running water maintained at the stated temperature. Before approximately 20 ml aliquots was poured out onto the sterile petridishes, the flask was shaken vigourously to mix the cellulose content properly with the molten agar. After the agar had solidified on the petridishes, stock cultures of common isolated fungi were inoculated by placing a uniform amount of agar block in the centre of each agar plate by means of a sterile wire loop. Four replicates were prepared for each culture inoculation, and plates were incubated by placing them in an incubator at 30°C for two weeks.

5.2.6. Inoculation on 1% Xylan agar

1% W/V Xylan was added into basal agar and autoclaved at 121°C for 20 minutes at 15/lb pressure. The other techniques employed were similar to the ones described in the previous section (see 5.2.2.).

5.2.7. Inoculation on Ball-milled straw added agar

1% straw(V/V)suspension was added into the basal agar medium; the other techniques employed were the same as reported in section 5.2.3.

5.2.8. Ligninolytic activities of isolated fungi

5.2.8.1. Bavendamms Polyphenoloxidase test on Gallic and Tannic Acid agars

Uniform amounts of pelleted inocula from stock cultures

were taken and placed at the centre of malt agars containing 0.5% gallic or tannic acids, respectively. For each inoculated fungus, 4 replicates were prepared, and the plates were incubated at 30°C for 4 weeks.

5.2.8.2. Inoculation on lignosulphonate added media

1% W/V lignosulphonate was incorporated into basal agar; the other details of the technique employed were the same as in Section 5.2.3.

5.2.8.3. Inoculation on Indulin A(R) containing agar

2% indulin A(R) (by weight) was placed in a 250 ml erlenmeyer conical flask containing a measured amount of distilled water. The contents were autoclaved at 121°C for 15 minutes at 15/lb pressure. After autoclaving, the sterilized aqueous suspension of lignin was found to contain a thin layer of dark material. According to the report of Day, Pelezar and Gottlieb's (1949) the formation of this material was a result of a thermoplastic change during autoclaving. Nevertheless this was removed from the flask aseptically. Day et al claimed that in the process of removing this layer of material, approximately 1% of the lignin was also removed. They noticed this after estimating the methoxyl content (Philips, 1934) from both the thin layer of material and from the lignin suspension left behind in the flask. Four replicates for each inoculum were made, and the plates were incubated at 30°C for two weeks.

5.2.8.4. Inoculation on Indulin A(R) containing liquid medium

The method used was similar to that indicated in Section 5.2.6.3., except that inocula were suspended in liquid medium containing 1% indulin A(R), placed in 100 ml erlemeyer conical flasks.

5.2.8.5. Inoculation on lignin agar prepared by 72% H_2SO_4 method

Inoculation and incubation methods employed were similar to those reported previously in section 5.2.6.3.

5.2.8.6. Inoculation on 1% lignin (prepared by 72% H_2SO_4 method) containing liquid medium

The method used for inoculation and incubation were similar to those reported in Section 5.2.6.5.

5.2.9. Assessment of growth response of inoculated fungi

Literature concerning cellulose degradation indicates that a variety of techniques have been developed by many workers to assess the cellulolytic activity of fungi (Siu, 1951; Gascoigne and Gascoigne, 1960 and Reese, 1963). Most of the techniques are based upon some artificially prepared form of cellulose. Reese (1946); Garrett (1962); Chang (1967) and others have employed a method of loss of dry weight of filter paper after fungal inoculation, while Hazra, Bose, and Guha (1958) used acid treatment for a rapid cellulose assaying technique. Malik and Eggins (1970) and Mills (1973) introduced a perfusion chromatography paper technique, which involves measuring the

loss of tensile strength.

Textile cotton has been assessed for fungal decomposition by the same method by Abrams (1950), Siu and Sinden (1951) and Allsopp (1973). In vitro, enzymic decomposition of cellulosic components was also studied by many investigators (Agarwal et al, 1963; Domsch and Gams, 1969; and Flannigan, (1970, 1972). Nisizawa (1963) reported the measurement of a decrease in viscosity of cellulose derivatives.

Measurement of clearance zones in cellulose agar was used by McBeth (1916), Scales (1916), Aschan and Norkrams (1953). Nevertheless, this technique was held to have certain limitations, because of the fact that some fungi tend to obscure the clearing zone. To solve this problem, Aschan and Norkrans (1953) incorporated a chemical, i.e. Merthiolat, in the agar to suppress the fungal growth and then measured the cellulolytic activity. A different approach was that of Walsh and Stewart (1969) who introduced a technique which involves measuring the intensity of clearing close to the fungus by counting the number of undissolved cellulose particles remaining in a specific area of the agar in a petridish after the apprearance of mycelial growth.

The technique selected in the present work was similar to the one described by Siu, R.G.H. (1951). During the incubation periods the petridishes were viewed against a daylight background and comparison was made with agar plates with no added carbon sources. Meanwhile, on what was planned to be the end of the incubation period, the clearing activities on agar plates supplemented with different carbon sources were recorded as 'very strong' (VS) or 'strong' (S) if the clearing zones were to cover approximately the entire surface area, and

more than half the petridishes, respectively. Moreover, the term 'moderate' (M), was used for the clearing of less than half the surface area, while clearing of well under half were regarded as definite (D). Finally, the term 'weak' was used for negligible clearing, and a minus sign was used to indicate no detectable activity on the agar.

Measurement of the growth on agar plates was made during the final days of incubation by measuring in centimetres the diameter of giant fungal colonies (Rege, 1927). It is noteworthy that measurement was made in two directions at right angles to each other. No attempt was made to measure the comparative thickness of the growth.

Measurement of fungal growth on liquid media was made by comparative visual observation recorded as a series of + or - signs according to whether or not any growth was evident (Days, Pelczerad Gottlieb, 1949).

5.3. RESULTS

5.3.1. Cellulolytic activities of inoculated fungi

The results are shown in Table 18 and on plates 1a, b and c for the cellulolytic activities and growth measurement of isolated fungi on Eggins and Pugh cellulose agar after incubation at 30°C. Each figure represents the average of 4 replicates.

Most of the inoculated fungi have developed their extensive growth by covering the entire surface area of the agar plate, but their cellulolytic activities were not evaluated clearly on the petridishes; this indicates that these fungi have obscured the clearance of incorporated cellulose with agar. This suggests the idea that it should be possible that the incoulated fungi have utilized other carbon containing compounds such as L-asparagine and yeast extract present in the medium. However, these fungi tended to show moderate clearing zones when viewed against the background of daylight and are tentatively identified as Alternaria sp. Aspergillus flavus, A. versicolor, Aspergillus sp., Cephalosporium sp. Fusarium sp.2, Myriococcum albomyces (thermophilic), Scopulariopsis brevicaulis and Stachybotrys atra. Nevertheless, no attempt was made at this level to assess their activities by growing them under strictly controlled conditions, where, apart from cellulose, no other carbon sources were supplemented in the growth medium. Some other fungi have also shown their moderate clearing activities on cellulose agar, e.g. Humicola sp., Paecimomyces sp. and Streptomyces sp. However, their growth measurements were also related to their clearing results, one can see from the figures and Table 18 that the colony diameters for those

Table 18. Growth and cellulolytic activity of isolated fungi after inoculation on Eggins and Pugh's cellulose agar medium at 30°C for two weeks incubation period

FUNGI	Colony diameter in centimetres	Cellulolytic activity
Alternaria tenuis	8.50	S
Alternaria sp.	8.50	M
Aspergillus fumigatus	8.50	S
A. flavus	8.50	M
A. versicolor	8.50	M
Aspergillus sp.	8.50	M
Aureobasidium pullulans	2.50	D
Botrytis cineria	6.00	W
Botryotrichum piluliferum	8.50	VS
Cephlosporium sp.	8.50	M
Chaetomium globosum	8.50	VS
Chaetomium sp.	8.50	S
Coprinus cinereus	8.50	VS
Fusarium sp.1.	8.50	S
Fusarium sp.2.	8.50	M
Gliocladium roseum	8.50	S
Humicola sp.	5.60	M
Myriococcum albomyces	8.50	M
Mucor sp.	4.00	W
Paecilomyces sp.	4.50	M
Penicillium sp.1.	3.20	W
Penicillium sp.2.	2.20	NA
Rhizopus sp.	2.90	NA
Scopulariopsis brevicaulis	8.50	M
S. chartarum	2.50	W
Scopulariopsis sp.	2.30	NA
Stachybotrys atra	8.50	M
Streptomyces sp.	4.30	M

VS: Very strong S: Strong M: Moderate
W: Weak NA No activity D: Definite

Note: Cellulolytic activity of fungi was measured on agar according to the method described by Siu, (1951).

Clearing of Cellulose or Xylan agar medium by selected fungi

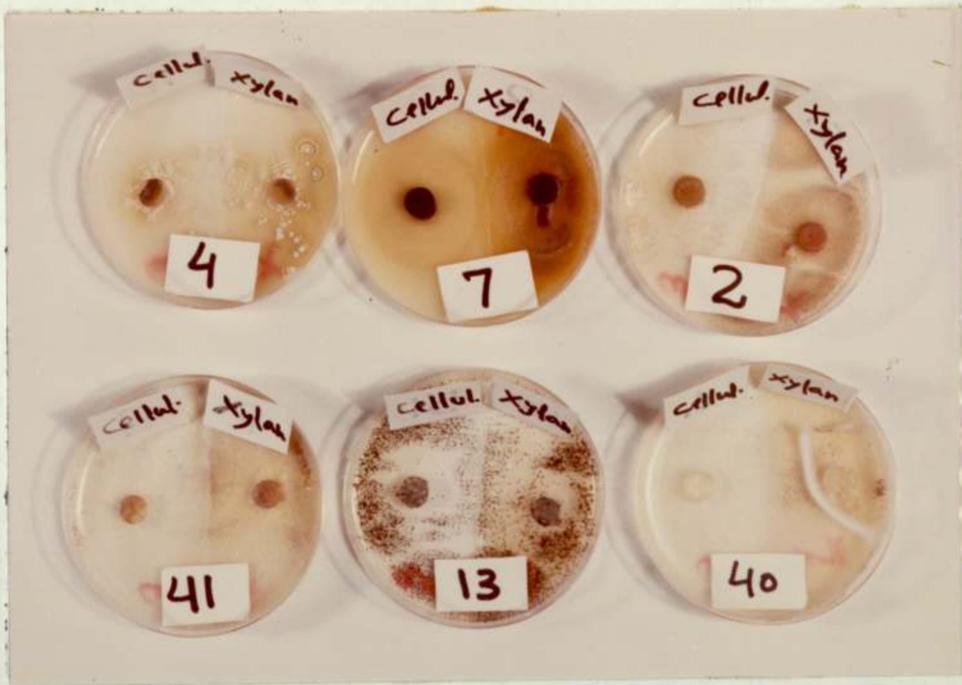


Plate 1 a*

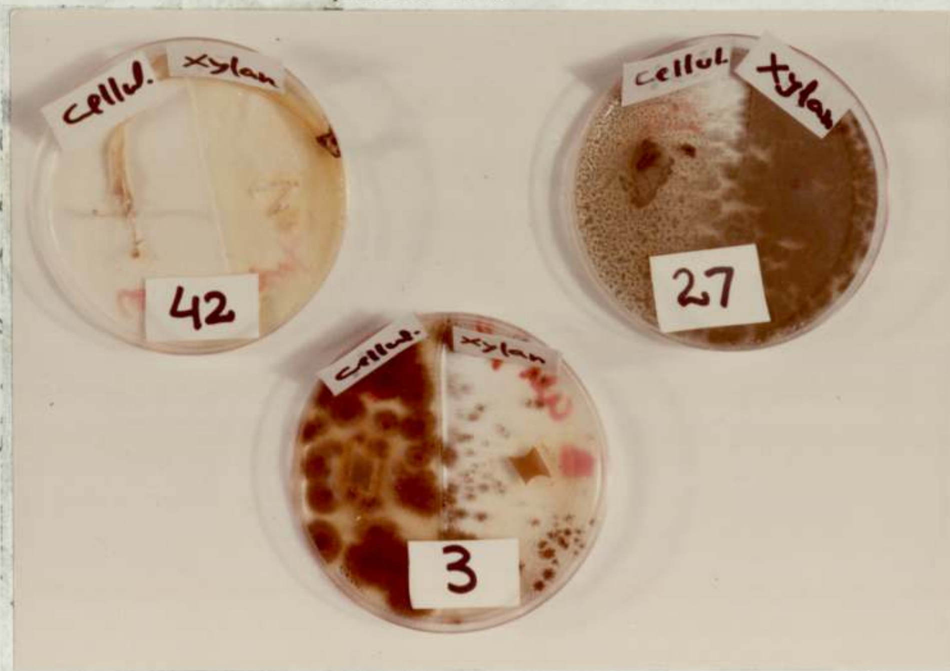


Plate 1 a* Plate 1 b**

4 = Streptomyces sp.

7 = Stachybotrys atra

2 = Aspergillus versicolor

41 = Coprinus cinereus (1)

Plate 1 a **

13 = Chaetomium globosum

40 = Coprinus cinereus

42 = Coprinus cinereus

27 = Aspergillus sp.

3 = Aspergillus sp.

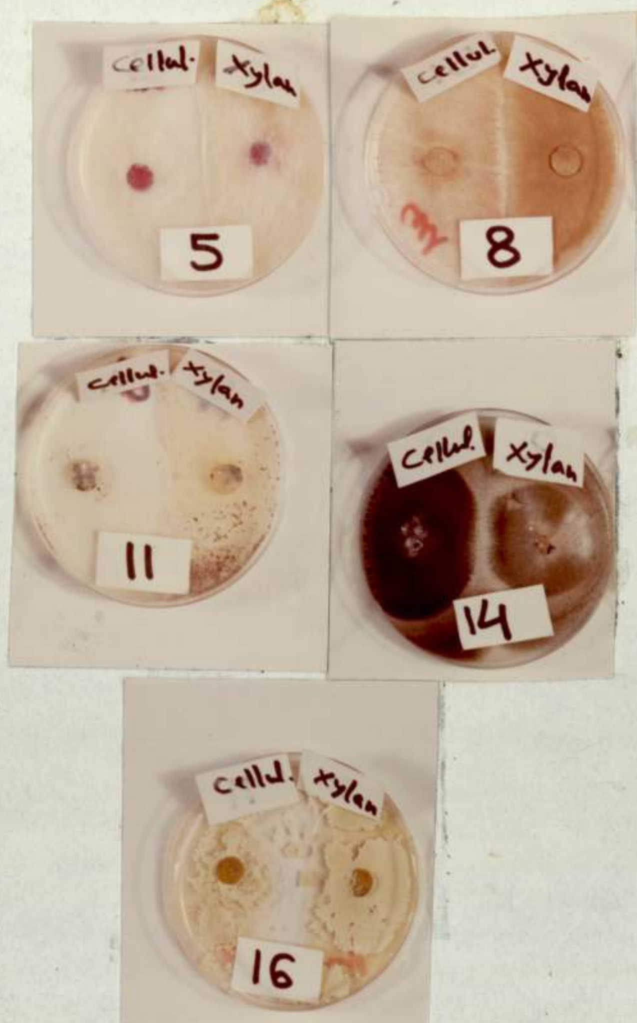


Plate I C

5 = Fusarium sp.

8 = Scopulariopsis brevicaulis

II= Myriococcum albomyces

I4= Alternaria tenuis

I6= Botryotrichum piluliferum

NB: Each petridish contains two types of agar medium: left hand side contains cellulose (cellulose:cellul.): right hand side contains xylan (xylan)

fungi were also extended moderatly, i.e. covering less than half or slightly more than half of the surface area of the agar plates. Only one species of fungus Aureobasidium pullulans showed a slight clearing around the central region of agar and was represented as definite (D) clearing. On the whole, a low percentage of weak or non cellulolytic fungi were recorded. The former ones were listed as Botrytis cineria, Cladosporium sp., Mucor sp., Penicillium sp and Scopulariopsis chartarum; where as the latter ones were listed as Penicillium sp 2 Rhizopus sp. and Scopulariopsis sp. However, growth rates of these fungi were also not promising as regards their cellulolytic activities; this could be seen easily by viewing their recorded colony diameters, which were found to be covering far less than half the surface area on agar plates.

Some fungi were recorded as highly cellulolytic on the basis of the criteria used here for judging their cellulose agar clearing activities. Although these fungi were already reported by many workers as highly cellulolytic in nature, but their activities were not measured under alkaline conditions of growth. However, present work showed the strong (S) cellulose clearing activities of Alternaria tenuis, Gliocladium roseum, A. fumigatus, Chaetomium sp. and Fusarium sp.1, and a very strong (VS) activity by Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus. All these above named fungi have shown their luxuriant growth by covering the entire surface areas on petri dishes, and the clearing of cellulose on agar was clearly visible. However, in comparison with ones showing strong activities, much more clearing and transparencies of cellulose agar plates were viewed by the ones

showing very strong activities. (Eastwood, 1952; Daniels, 1961; Pugh et al, 1963; Domschand Gams, 1969; Flannigan, 1969; Chang and Hudson, 1969 and Flannigan, 1970, 1972).

5.3.2. Xylanolytic activities of isolated fungi

Results are given in Table 19 for the xylan utilisation of isolated fungi, taking the average of four replicates from each fungus. (Clearing shown in plates 1a, 1b and 1c).

More encouraging results were obtained after recording the xylanolytic activities of isolated fungi in comparison with the cellulolytic activities. It is also noteworthy that instead of adding any carbon containing compounds, xylan was used as the sole carbon source in the basal agar medium. The fungi which were showing moderate cellulolytic activities and less growth by previous inoculation method (see Table 18) showed more clearing of xylan on agar and comparatively more extension in their colony diameters. These fungi could be listed as Alternaria sp., Botrytis cineria, Gliocladium roseum, Myriococcum albomyces and Stachybotrys atra. The remaining fungi such as Alternaria tenuis, Chaetomium sp. and Fusarium sp. have given nearly the same results as recorded in the previous section on cellulolytic activities (5.3.1).

In the same manner, the fungi Aspergillus versicolor Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus have shown their luxuriant growth and very strong xylanolytic activities on agar plates. A fumigatus has upgraded its value by showing characteristics similar to those obtained with the above named fungi.

Moderate clearing and growth rates were revealed by A

Table 19. Growth of isolated fungi on basal agar medium containing 1% xylan as the sole carbon source after two weeks incubation at 30°C

FUNGI	Colony diameter in centimetres	Xylanolytic activity
Alternaria tenuis	8.50	S
Alternaria sp.	8.50	S
Aspergillus fumigatus	8.50	S
A. flavus	8.50	S
A. versicolor	8.50	VS
Aspergillus sp	6.20	M
Botrytis cineria	8.50	S
Botryotrichum piluliferum	8.50	VS
Cephalosporium sp	8.50	M
Chaetomium globosum	8.50	VS
Chaetomium sp.	8.50	S
Cladosporium sp.	5.20	M
Coprinus cinereus	8.50	VS
Fusarium sp.1.	8.50	S
Fusarium sp.2.	8.50	S
Gliocladium roseum	8.50	S
Humicola sp.	6.80	S
Myriococcum albomyces	8.50	S
Mucor sp.	8.50	M
Paecilomyces sp.	5.80	M
Penicillium sp.1.	4.20	W
Penicillium sp.2.	4.00	NA
Rhizopus sp.	4.90	W
Scopulariopsis brevicaulis	8.50	M
S. chartarum	7.00	D
Scopulariopsis sp.	5.20	W
Stachybotrys atra	8.50	S
Streptomyces sp.	4.80	M

VW: Very Strong S: Strong M: Moderate
D: Definite W: Week NA: No Activity

Note: Xylanolytic activity of fungi have been measured on agar according to the method described by Siu, (1951)

flavus, Aureobasidium pullulans, Cephalosporium sp., Cladosporium sp., Mucor sp., Paecilomyces sp., Scopulariopsis sp and Streptomyces sp. This shows that types of growth rates and clearing zones were obtained similar to those found in the assessment of cellulolytic activities. Nevertheless, the fungus Aureobasidium pullulans, Cladosporium sp. and Mucor sp. have seemed to upgrade their values after showing better utilization of xylan by evaluating more clearing and considerable extension in their colony diameters.

Apart from the better utilisation of xylan by most of the fungi, as outlined above, some inoculated fungi were still showing no growth and clearing activities or at least their activities were negligible on xylan agar plates. These include: Penicillium sp.1 and 2, and Scopulariopsis sp. Nevertheless, comparing these fungi, a better result was given by Scopulariopsis chartarum by showing a definite clearing.

5.3.3. Fungal growth and activities on ball-milled straw added agar

Table 20 shows the results of assessing the clearing of 1% ball-milled straw added agar and its utilization by inoculated fungi. The very strong clearing on agar was revealed by Botryotrichum piluliferum and Coprinus cinereus, while strong activities were shown by Alternaria tenuis, A. fumigatus, Chaetomium globosum and Myriococcum albomyces. These fungi covered the entire surface area on petri dishes. Although other fungi have also shown such vigorous growth, their clearing activities were not very obvious; therefore their activities were tentatively recognized as moderate. These fungi were shown in Table 20 as Alternaria sp., A. flavus, A versicolor, Cephalosporium sp., Chaetomium sp., Gliocladium roseum, Fusarium sp 1 and 2, Scopulariopsis sp. and Stachybotrys atra. Here it is worth noting that, due to the dense colonisation of ball-milled straw, it was fairly difficult to evaluate the clearing abilities of colonised fungi on agar plates. However, comparisons were made by, using un-inoculated agar plates as controls when observations were made against the daylight background. In addition to that, the petridishes were viewed through a magnified hand lens and also by placing the dishes up-side-down on an illuminated bench lamp.

A larger number of inoculated fungi showed their weak or negligible clearing activities but showed vigorous growth by extending colony diameters constantly on the surface area on petridishes. Among those fungi the most common ones were Mucor sp., Rhizopus, along with others like Botrytis cineria, Humicola sp., Paecilomyces sp., S.chartarum, Scopulariopsis sp

Table 20. Growth of isolated fungi on basal medium containing 1% ball-milled straw suspension as the sole carbon source after two weeks incubation at 30°C

FUNGI	Colony diameter in cenimetres	Clearance on agar medium
Alternaria tenuis	8.50	S
Alternaria sp.	8.50	M
Aspergillus fumigatus	8.50	S
A. flavus	6.50	M
A. versicolor	8.50	M
Aureobasidium pullulans	3.00	W
Botryotrichum piluliferum	8.50	VW
Botrytis cineria	7.50	W
Cephalosporium sp.	7.00	M
Chaetomium globosum	8.50	S
Chaetomium sp.	8.50	M
Coprinus cinereus	8.50	VW
Cladosporium sp	3.30	W
Fusarium sp.1	6.50	M
Fusarium sp.2.	7.20	M
Gliocladium roseum	8.50	M
Humicola sp.	5.00	W
Myriococcum albomyces	8.50	S
Mucor sp	8.50	S
Paecilomyces sp.	4.30	W
Penicillium sp	3.00	NA
Rhizopus sp	8.50	NA
Scopulariopsis brevicaulis	8.50	M
S. chartarum	5.60	NA
Scopulariopsis sp.	3.90	NA
Stachybotrys atra	8.50	M
Streptomyces sp.	8.50	W

VS: Very strong, S: Strong, M: Moderate
D: Definite, W; Weak NA: No activity

Note: Clearing of ball-milled straw containing agar was recorded according to the method described by Siu(1951)

and Streptomyces sp., which were showing comparatively less colony extensions. Far less than half or negligible coverage of agar plates was shown by Aureobasidium pullulans and Penicillium sp 1 and 2.

As far as the fungal colonisation rates were concerned, more extensive growth appeared when ball-milled straw was used in comparison, when cellulose or xylan were used as the carbon sources. Clearing activities in contrast were not rapid under these conditions for most of the fungi. These fungi have however, shown distinct clearing zones on than agar when the last two methods of inoculation were employed.

5.3.4. Bavendamm's Polyphenoloxidase reaction and fungal growth on 0.5% Gallic or Tannic-Acid agar plates

Results are shown in Tables 21, 22 and 23, using averages of the 4 replicates for each fungus. They are also illustrated on some photographs (i.e. plates 2a, b, c and d).

After two weeks' incubation, the petridishes were viewed against a daylight background. A comparison was made for each fungus using control plates without any supplementation of phenolic compounds. It was noticed that brown to dark brown diffusion zones appeared under and around the fungal mycelial mats on agars of gallic or tannic acid. This indicated Bavendamm's polyphenol oxidase positive reaction, and the fungi involved were found to be *Aspergillus* sp. *Botryotrichum piluliferum*, *Chaetomium globosum*, *Chaetomium* sp., *Penicillium* sp. 1 and *Stachybotrys atra*. Some other fungi, such as *A. flavus* and *A.versicolor* also showed light to mid brown diffusion zones, which wereonly visible from the underside of the petridishes. The former one showed this reaction on gallic acid medium, whereas the latter one was on tannic acid. (See Table 21). It was somewhat tedious comparing these fungi with their control plates, because they were previously reported as dark coloured fungi (Raper and Fennell, 1965). However, the same difficulties were also faced by Davidson et al (1938) and they eventually suggested that these sorts of fungi could be considered as negative on Bavendamm's scale. The rest of the fungi showed no clear reaction at all on both of the phenolic acid supplemented agars, even after four weeks of incubation. In contrast, the intensity of colour formation was increased gradually with the prolonged period of incubation for the Bavendamm's positive fungi described above.

Table 21. Bavendamm's polyphenoloxidase reaction of isolated fungi after inoculation on gallic acid and tannic acid agar media, and incubated at 30°C for two weeks

Fungi	Colour reaction on gallic acid	Colour reaction on tannic acid
Alternaria tenuis	-	-
Alternaria sp.	-	-
Aspergillus fumigatus	-	-
A. flavus	+	-
A. versicolor	-	+
Aspergillus sp.	4+	3+
Aureobasidium pullulans	-	-
Botrytis cineria	-	-
Botryotrichum piluliferum	5+	2+
Cephalosporium sp.	-	-
Chaetomium globosum	5+	5+
Chaetomium sp.	2+	2+
Coprinus cinereus	-	-
Fusarium sp.1	-	-
Fusarium sp.2	-	-
Gliocladium roseum	-	-
Humicola sp.	-	-
Myriococcum albomyces	-	-
Mucor sp.	-	-
Paecilomyces sp.	-	-
Penicillium sp.1.	2+	3+
Penicillium sp.2.	-	-
Rhizopus sp.	-	-
Scopulariopsis brevicaulis	-	-
S. chartarum	-	-
Scopulariopsis sp.	-	-
Stachybotrys atra	5+	5+
Streptomyces sp.	-	-

(See Table 22 for the signs)

Note: Colour ractions have presented using the method adapted by Davidson, Campbell and Blaisdell (1938)

Table : 22 This chart is based on the observations of Davidson, *et*. *al*. (I938)

Bavendamm's polyphenoloxidase reaction on phenolic acid media	
+ =	Diffusion zone light to dark brown, formed under inoculum at the centre of dish. In case no growth takes place a faint brown discolouration under the inoculum.
2 + = + +	Diffusion zone light to dark brown formed under most of mycelial mats but not extending to margin. Visible from underside only.
3 += + + +	Diffusion zone light to dark brown, extending a short distance beyond the margin of the mat and visible from upperside.
4 +=+ + + +	Diffusion zone dark brown , opaque, extending a short distance beyond the margin of fungus mat.
5 + = +++++	Diffusion zone very intensive, dark brown, opaque, forming a wide corona about the mycelial mats.

Table 23. Colony diameter of inoculated fungi on gallic acid and tannic acid agar media after two weeks incubation at 30°C

FUNGI	Mycelial diameter in centimetres	
	On gallic acid	On tannic acid
Alternaria tenuis	7.50	6.80
Alternaria sp.	7.50	6.40
Aspergillus fumigatus	3.50	2.50
A. flavus	8.50	8.50
A. versicolor	8.50	8.50
Aspergillus sp.	7.60	5.40
Aureobasidium pullulans	2.00	1.50
Botrytis cineria	3.50	2.00
Botryotrichum piluliferum	7.50	1.00
Cephalosporium sp.	2.50	1.00
Chaetomium globosum	7.70	6.50
Chaetomium sp.	5.40	2.50
Coprinus cinereus	-	-
Cladosporium sp.	-	-
Fusarium sp.1.	6.50	4.50
Fusarium sp.2.	6.50	2.51
Gliocladium roseum	2.40	-
Humicola sp.	-	-
Myriococcum albomyces	-	-
Mucor sp.	-	-
Paecilomyces sp.	2.20	-
Penicillium sp.1.	5.50	3.10
Penicillium sp.2.	3.10	1.50
Rhizopus sp.	-	-
Scopulariopsis brevicaulis	3.10	2.50
S. chartarum	2.10	1.40
Scopulariopsis sp.	1.00	-
Stachybotrys atra	2.50	2.00
Streptomyces sp.	-	-

Bavendamm's Polyphenoloxidase Positive Reaction

On gallic acid agar medium

Plate Number: 2 a

On tannic acid medium

Plate Number 2 b

Plate Number 2 a *

7 = Stachybotrys atra

3 = Botryotrichum-piluliferum

27= Aspergillus sp.

2 = Penicillium sp.

13= Chaetomium globosum

Bavendamm's Polyphenoloxidase Negative Reaction

On gallic acid agar medium

Plate number 2c

On tannic acid agar medium

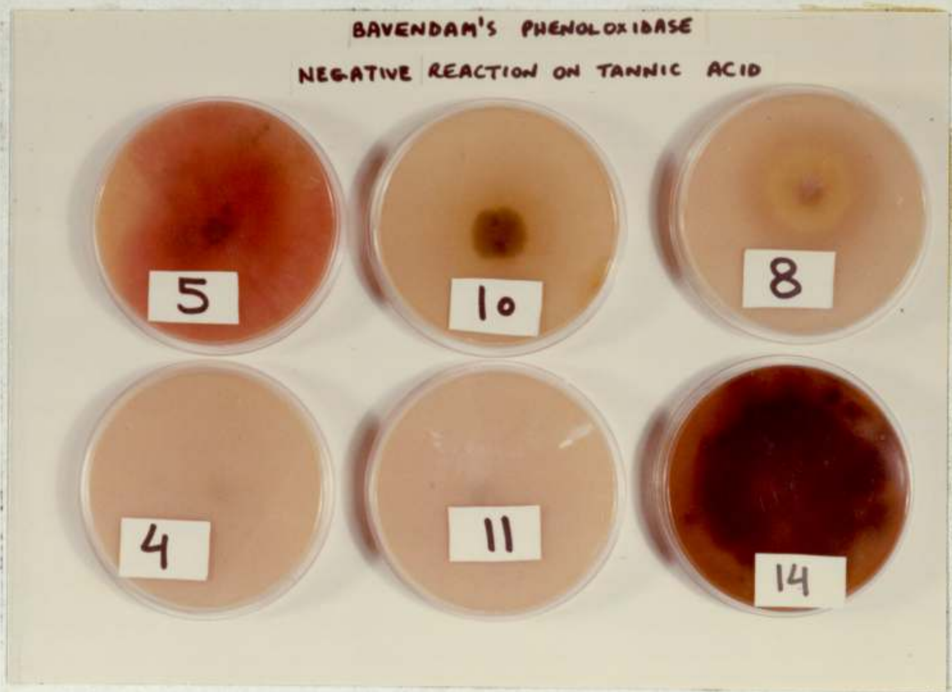


Plate number 2d

5= *Fusarium* sp.
IO= *Aureobasidium pullulans*
8= *Scopulariopsis brevicaulis*
4= *Streptomyces* sp.
II= *Myriococcum albomyces*
I4= *Alternaria tenuis*

Examining fungal colonisation on phenolic acid supplemented agars, it was found that the most vigorous growth was shown by A. flavus and A. versicolor which covered the entire surface area of the agar plates. Alternaria tenuis, Alternaria sp., Fusarium sp.1 and 2, Penicillium sp.1, on the other hand, showed their growth by covering more than half of the surface area on the agar plates. However, it is important to bear in mind that all these fungi were tentatively showing negative reactions.

Fungi with positive reactions also showed active growth, except for the negative growth of Botryotrichum piluliferum and Chaetomium sp. on gallic and tannic acid agar plates respectively, (see Table 23).

5.3.5. Ligninolytic activities of inoculated fungi

Results are summarised in Table 24, and in Figure 2, for the growth of a selected number of fungi using different lignins as the sole carbon sources in the solid and liquid media. It could be seen from figure 2 that the utilization of lignosulphonate was more widespread in comparison with other lignin sources, and the most extensive growth was shown on this medium by the fungus Botryotrichum piluliferum, Chaetomium globosum, Chaetomium sp., Humicola sp., Myriococcum albomyces, Scopulariopsis brevicaulis, Stachybotrys atra and Streptomyces sp. No attempt was made to measure the comparative thickness of the growing fungal colonies. However, the colony diameters were recorded in cm. Some of the fungi such as Alternaria tenuis, Coprinus cinereus, and Fusarium sp. gave indications of being inhibited by the presence of lignin sources in their growth media by not

Table 24. Growth of a selected number of fungal isolates on basal agar media containing 1% lignin as the sole carbon source, after incubation at 30°C for ten days.

FUNGI	Mycelial diameter in centimetres		
	1% lignosulphonate	1% Indulin A(R)	1% lignin prepared by 72% H_2SO_4 method
Alternaria tenuis	-	-	-
Aspergillus fumigatus	2.10	2.00	1.00
A. versicolor	2.40	1.80	1.20
Botryotrichum piluliferum	8.50	7.00	5.00
Chaetomium globosum	8.50	8.00	7.50
Chaetomium sp.	8.30	4.40	3.90
Coprinus cinereus	-	-	-
Fusarium sp.l.	-	-	-
Gliocladium roseum	6.60	4.60	4.50
Humicola sp.	8.50	7.80	6.50
Myriococcum albomyces	7.00	3.10	5.00
Scopulariopsis brevicaulis	5.60	2.50	2.00
Stachybotrys atra	5.50	3.50	4.40
Streptomyces sp.	4.40	2.10	1.30

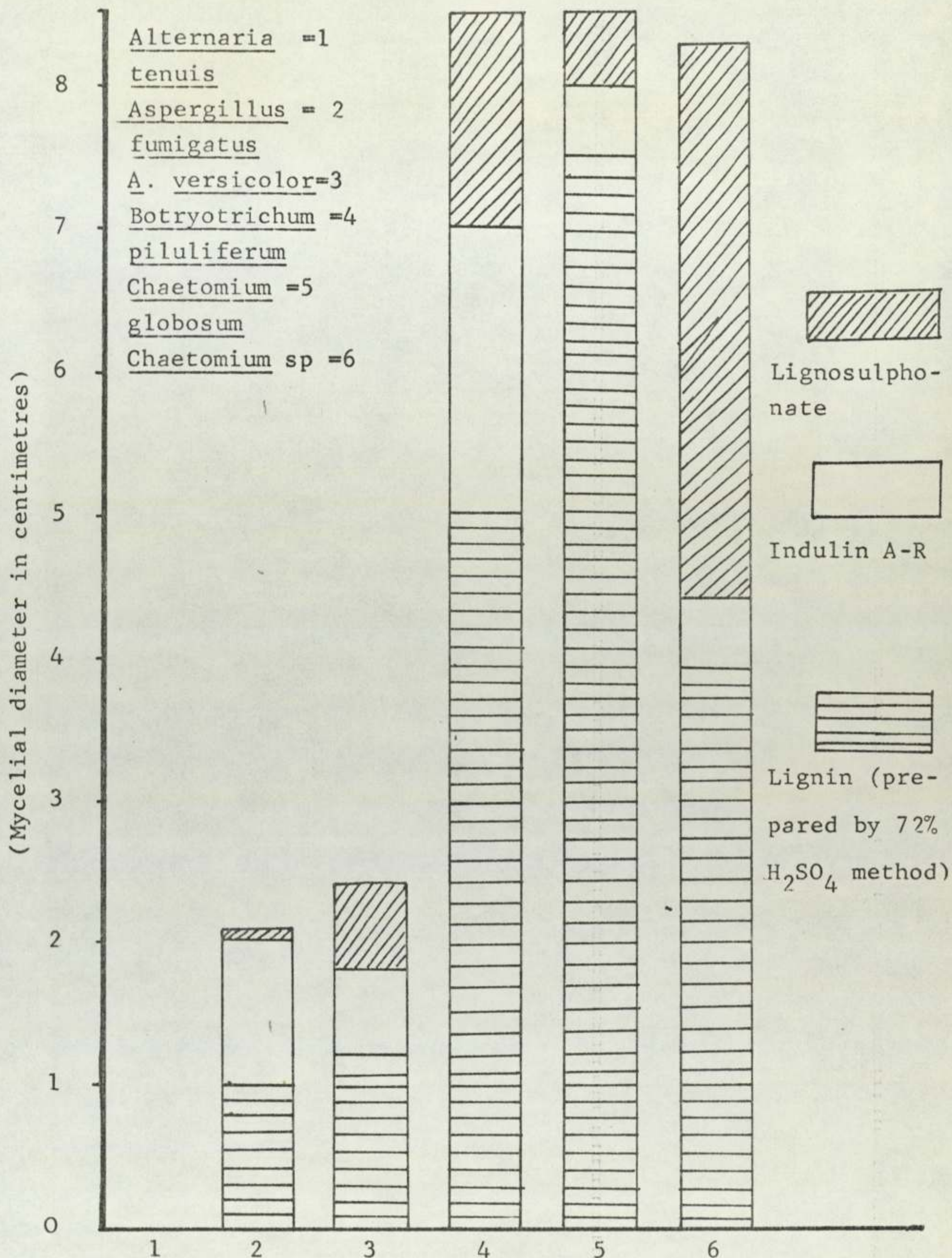


Figure: 2 Measurement of mycelial growth of isolated fungi on basal agar media containing lignins as the sole carbon sources

NB: Fungi are represented in numerical order.

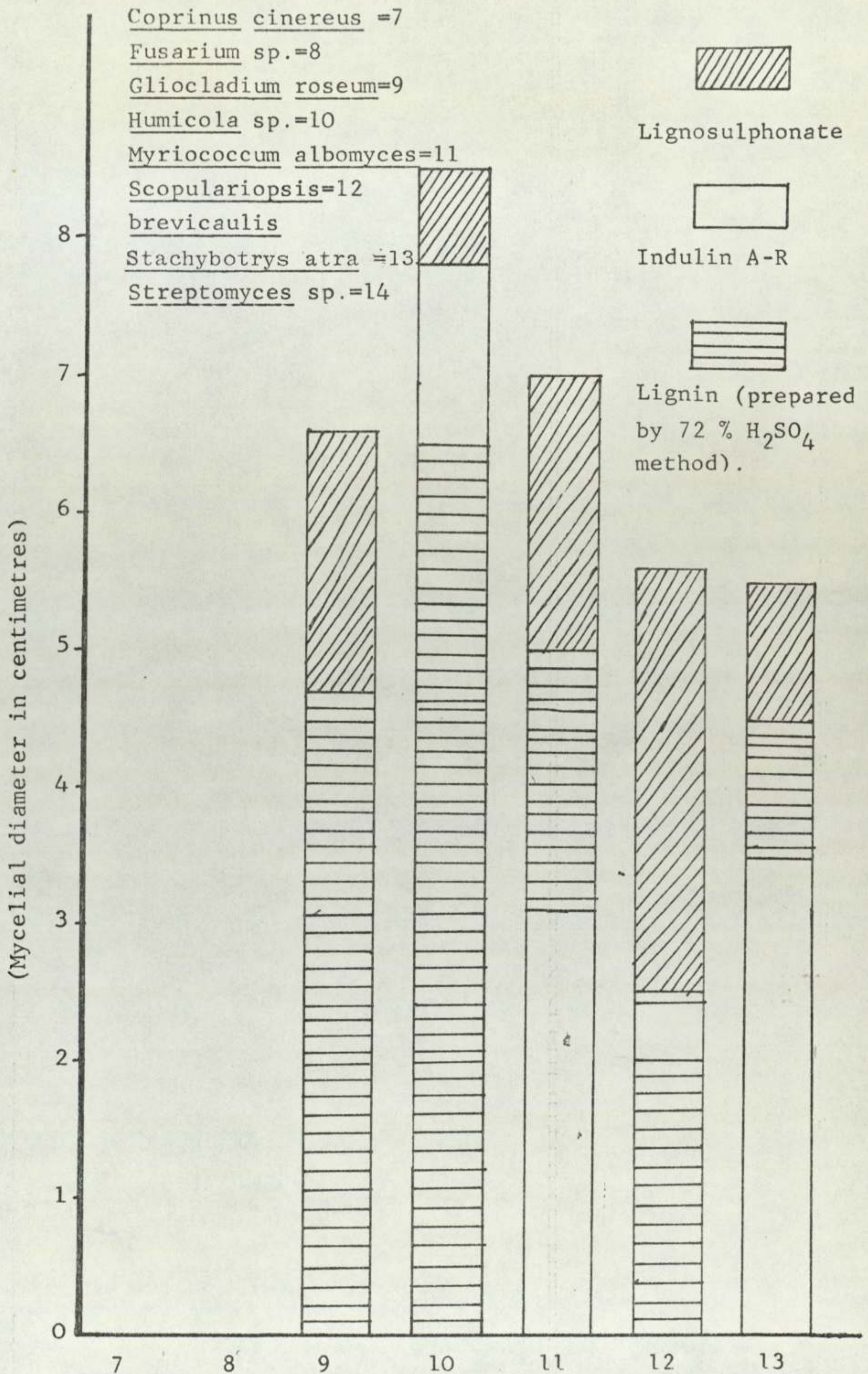


Figure 2 (cont.)
Coprinus cinereus =7
Fusarium sp.=8
Gliocladium roseum=9
Humicola sp.=10
Myriococcum albomyces=11
Scopulariopsis=12
brevicaulis
Stachybotrys atra =13
Streptomyces sp.=14
Lignosulphonate
Indulin A-R
Lignin (prepared
by 72 % H_2SO_4
method).
(Mycelial diameter in centimetres)
0
1
2
3
4
5
6
7
8
7
8
9
10
11
12
13

showing any evidence of their growth on all three lignins added in agar media. On the other hand, Lendingham and Adams (1942) have reported the growth of Alternaria tenuis on lignosulphonate medium, while Fischer (1933) reported the growth of this species on Phenol lignin. Moreover, some of the Coprinus species were also reported to be ligninolytic by Fries (1955): she also measured the enzyme ligninase activities of C.fimetarius, C.curtis, C.ephemerus, C.tardus, C.naroticus, C.atramentasium and C.comatus and tentatively classified all of them by giving positive reactions. It is noteworthy that one of the species, i.e. C.fimetarius was supposed to be recognised as C.cinereus (buller, 1931; Pinto-Iopes, 1971). Most of the species of Fusarium were also reported to be utilizing lignosulphonate by Sukhenko et al (1951). Gulyas (1967) has also reported the degradation of 20% wheat straw lignin by some Fusarium spp.

Looking at the results from shake and stationary culture incubation (see Tables 25a and b), it could be seen that the fungi Botryorichum piluliferum and Chaetomium globosum were showing promising growth response on both incubation methods referred to, but the former fungus was producing more mycelial pellets from stationary culture while inoculation was made in indulin A(R) and lignin prepared by 72% H_2So_4 method (Ellis, Matrone and Maynard, 1946). Some inoculated fungi like A. fumigatus and A. versicolor were giving better growth response on stationary culture rather than shake culture incubation, as it is believed that the latter method could provide more aeration and availability of suspended lignin to use as the sole carbon source. However, the fungus Chaetomium sp. was

Table: 25 a

Stationary culture inoculation of a selected number of fungi in basal media containing 1 % lignin as the sole carbon sources after incubation at 30°C. for ten days

FUNGI	Fungal growth on liquid surface		
	1 % lignosul·	1 % Indulin A-R	1 % lignin prepared by 72 % H_2SO_4 method
Alternaria tenuis	−	−	−
Aspergillus fumigatus	4+	2+	2+
A. versicolor	4+	2+	2+
Botryotrichum piluliferum	4+	4+	4+
Chaetomium globosum	4+	4+	4+
Chaetomium sp.	2+	2+	2+
Coprinus cinereus	−	−	−
Gliocladium roseum	2+	3+	2+
Fusarium sp.	−	−	−
Humicola sp.	−	−	−
Myriococcum albomyces	−	±	−
Scopulariopsis brevicaulis	3+	4+	2+
Stachybotrys atra	4+	2+	2+
Streptomyces sp.	+	4+	2+

− , indicates no growth ; ± , doutful growth response ; + , 25 % liquid surface covered with fungal growth ; 2+ , 50 % liquid surface covered ; 3+ , 75 % liquid surface covered ; 4+ , 100 % liquid surface covered

Table : 25 b

Shake culture inoculation of a selected number of fungi in basal media containing 1 % lignin as the sole carbon source, after incubation at 30°C. for ten days.

FUNGI	Fungal growth on liquid surface		
	1 % lignosul-phonate	1 % Indulin A-R	1 % lignin prepared by 72 % H_2SO_4 method
Alternaria tenuis	–	–	–
Aspergillus fumigatus	2+	+	+
A. versicolor	2+	±	+
Botryotrichum piluliferum	4+	2+	2+
Chaetomium globosum	4+	4+	4+
Chaetomium sp.	2+	+	2+
Coprinus cinereus	–	–	–
Gliocladium roseum	4+	4+	4+
Fusarium sp.	–	–	–
Humicola sp.	+	+	–
Myriococcum albomyces	±	±	±
Scopulariopsis brevicaulis	4+	3+	4+
Stachybotrys atra	4+	2+	2+
Streptomyces sp.	+	4+	2+

– , indicates no growth; ±, doubtful growth response; + , % liquid surface covered with fungal pellets; 2+ , 50 % surface covered ; 3+ , 75 % surface covered; 4+ , 100 % surface covered.

evaluated as having a similar growth response by both incubation techniques except that there was a 25% (+) surface coverage by shake culture incubation when inocubation was made on indulin A (R). The fungus, Gliocladium roseum gave a better growth response with shake culture incubation, as it utilized lignins added as the only carbon sources. In addition to that, a similar result was recorded for Scopulariopsis brevicaulis, Stachybotrys atra and Streptomyces sp. In constrast, negative growth response was observed for Alternaria tenuis, Coprinus cinereus, Fusarium sp. and Humicola sp., though the last-named fungus showed a growth response on media containing lignosulphonate when incubation was made on shake culture. It is interesting to note that although Humicola sp. showed Bavendamm's negative reaction, but good growth was recorded by this fungus when inoculation was made on all lignins in agar plates, particularly the coverage of entire agar plate was noticed when lignosulphonate was added.

5.4. UTILIZATION OF XYLAN AND CELLULOSE AS THE SOLE CARBON SOURCES BY SOME OF THE SELECTED FUNGAL ISOLATES AT A RANGE OF ALKALINE pH LEVELS

5.4.1. Experimental procedure

Three sets of xylan (1%) agar media were prepared by the method described in Section 5.2.6., and the pH values of these media were adjusted to 7.5, 8.5 and 9.5 using sorensen phosphate buffer solutions (Lindeberg, 1939, 1944). In addition to that, a similar method was employed for the preparation of 1% ball-milled cellulose agar media, the pH being adjusted to the above mentioned ranges. Nevertheless, instead of using the components of Eggins and Pugh's (1962) cellulose agar medium (as used in previous experiments) a basal medium was added with cellulose. This medium contained no other carbon sources such as L.asparagine or yeast extract, only the added cellulose in it.

For inoculation and incubation of four different fungi, i.e. *Aspergillus fumigatus*, *Botryotrichum piluliferum*, *Chaetomium globosum* and *Coprinus cinereus*, similar procedures were employed to those mentioned in section 5.2.5 for assessing the xylanolytic and cellulolytic activities of isolated fungi.

5.5. RESULTS

Results are represented in tabular form and in graphs (see table 26a,b ; Figures 3a and b). The illustrated graphs also showed that the fungal growth on xylan containing agars was more abundant than that on cellulose-supplemented agars. This shows that the xylan decomposing abilities of the

Table 26a

Growth measurement of some selected fungi in centimetres after inoculation on xylan-supplemented agars adjusted at 3 alkaline pH ranges after 10 days of incubation at 30°C

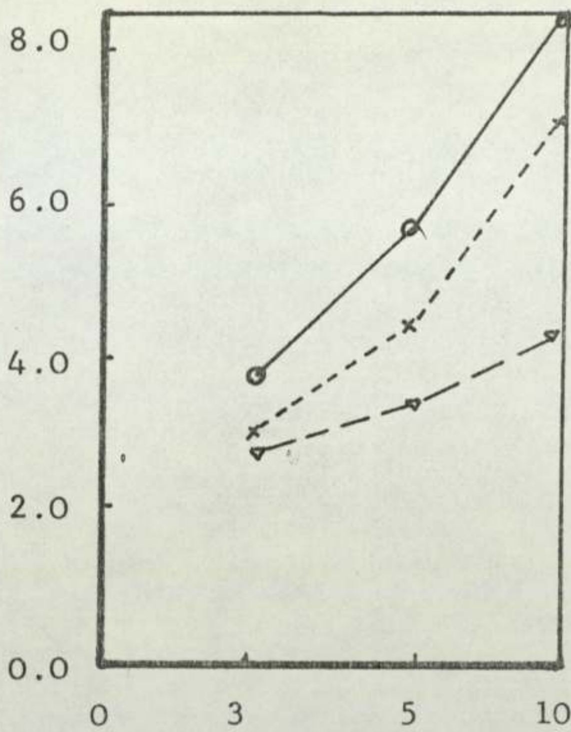
Fungi	Growth on 1% xylan agars, adjusted to three different alkaline pH levels after 3, 5 and 10 days.								
	ph: 7.5			pH: 8.5			pH: 9.5		
	3	5	10	3	5	10	3	5	10
Aspergillus fumigatus	3.0	4.5	8.5	2.5	3.8	7.8	1.8	2.5	3.5
Botryotrichum piluliferum	5.0	7.8	8.5	4.0	5.8	8.5	3.2	3.8	4.8
Chaetomium globosum	5.8	7.8	8.5	4.5	6.1	8.5	3.0	5.5	7.0
Coprinus cinereus	5.5	8.0	8.5	4.8	7.5	8.5	4.0	6.0	7.0

Table 26b

Growth measurement of some selected fungi in centimetres after inoculation on 1% cellulose supplemented agars adjusted at three different alkaline pHs, after incubation at 30°C for 10 days

Fungi	Growth on 1% cellulose agars, adjusted to three different alkaline pH levels after 3, 5 and 10 days								
	pH: 7.50			pH:8.5			pH.9.50		
	3	5	10	3	5	10	3	5	10
Aspergillus fumigatus	2.8	4.2	8.5	2.6	3.5	5.5	2.12	2.2	2.8
Botryotrichum piluliferum	3.8	6.5	8.5	3.0	4.5	7.0	2.90	3.3	4.2
Chaetomium globosum	4.8	7.2	8.5	3.5	7.0	8.5	2.5	5.0	7.2
Coprinus cinereus	3.8	7.8	8.5	3.5	6.9	8.5	2.8	5.0	7.2

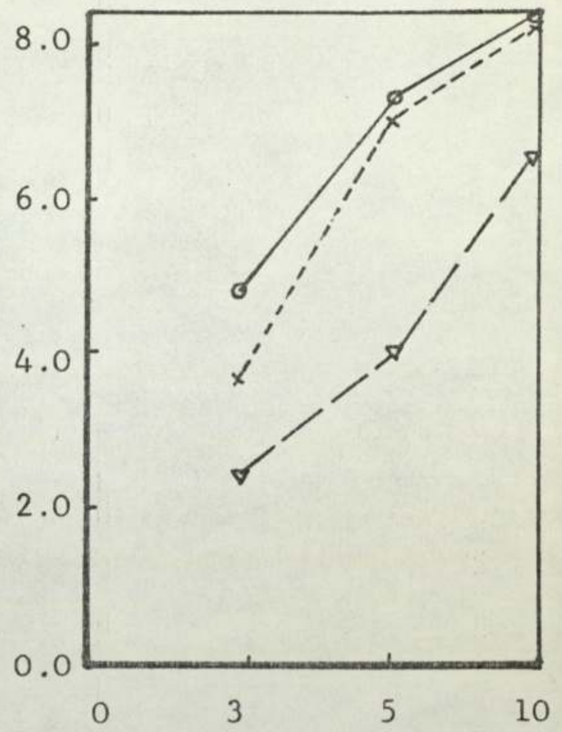
Mycelial diameter in cm.



(Days of incubation)

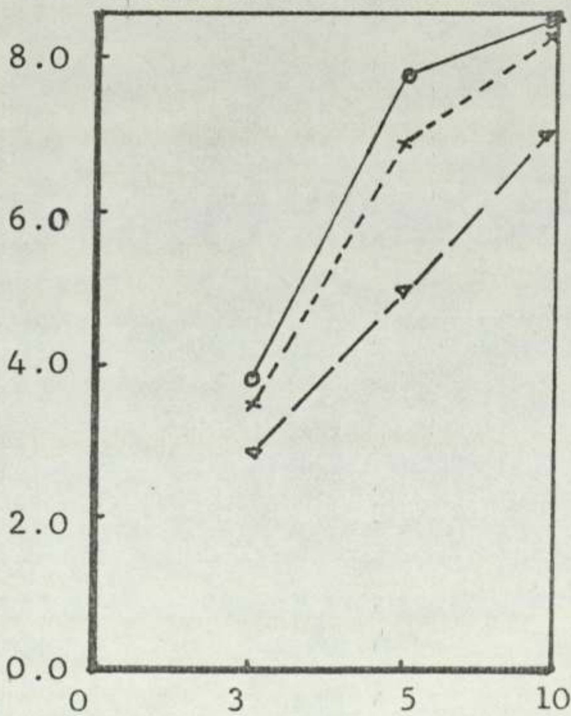
Botryotrichum piluliferum

Mycelial diameter in cm.



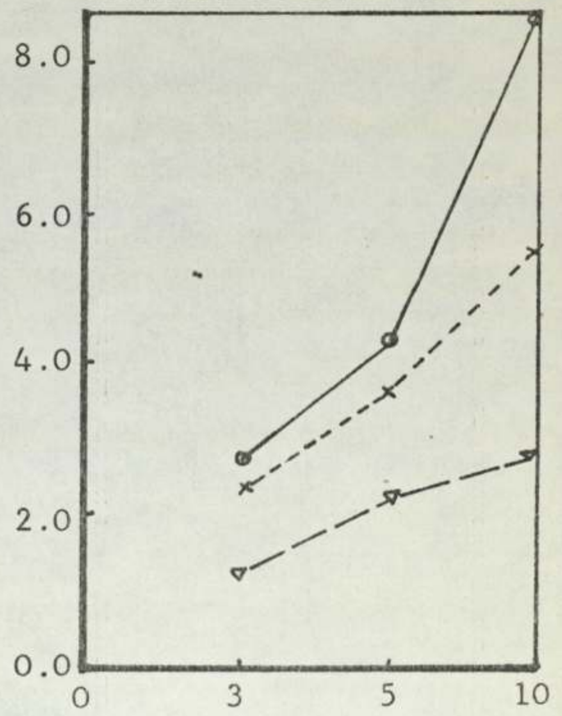
(Days of incubation)

Chaetomium globosum



(Days of incubation)

Coprinus cinereus



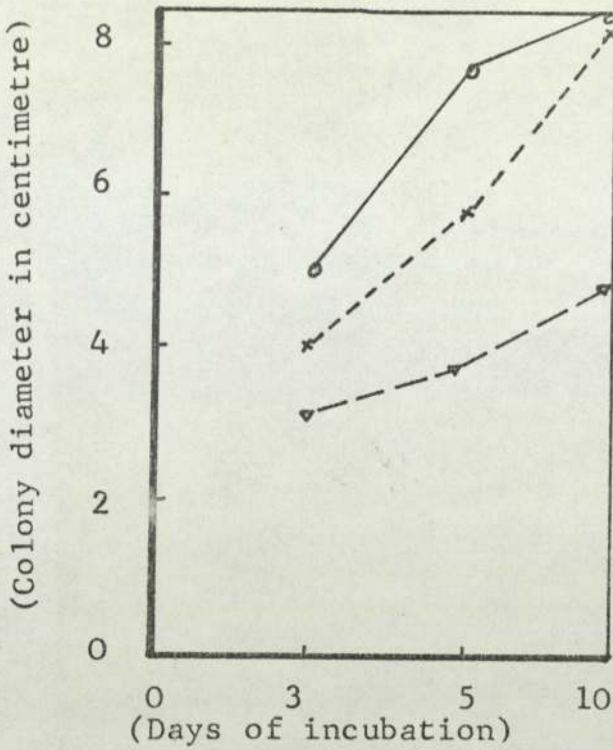
(Days of incubation)

Aspergillus fumigatus

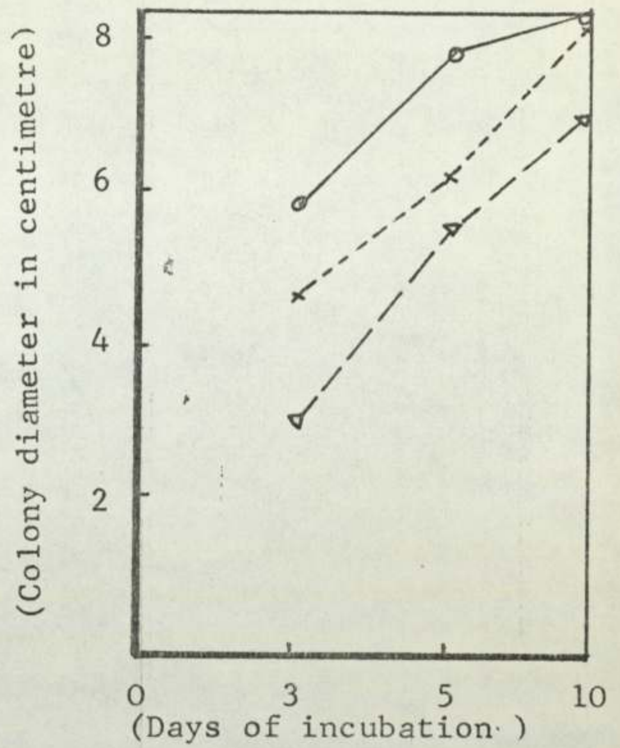
Figure:3 a Growth of fungi on cellulose agar media adjusted at a range of alkaline pH

O———O = pH 7.5; x-------x = pH 8.5; Δ– – – – –Δ = pH 9.5

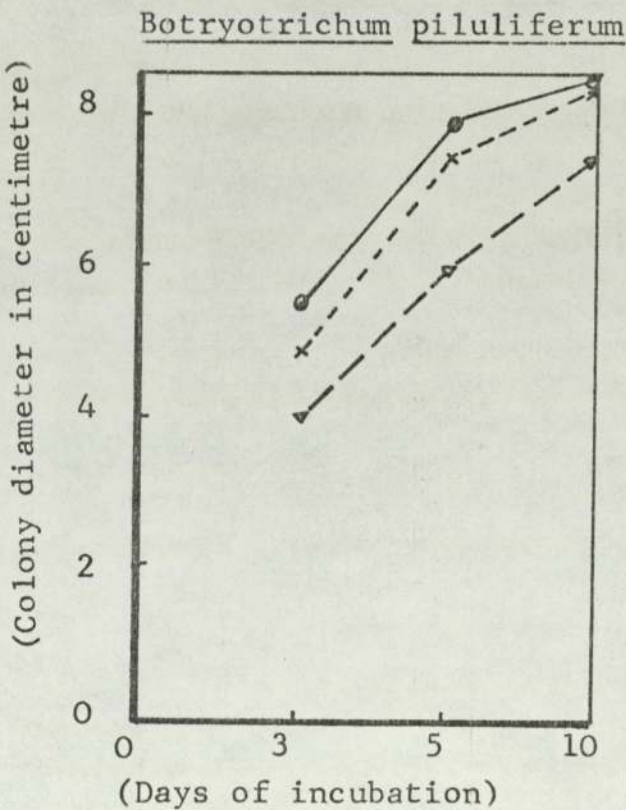
Figure: 3b Growth of fungi on xylan agar media adjusted at a range of alkaline pH



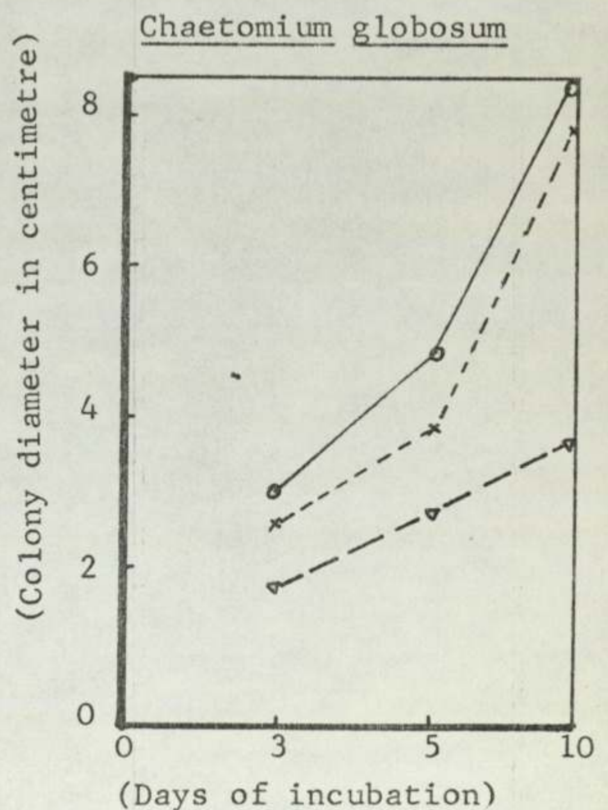
Botryotrichum piluliferum



Chaetomium globosum



Coprinus cinereus



Aspergillus fumigatus

O———O =pH 7.5; X- - - - -X =pH 8.5; Δ— — —Δ =pH 9.5

inoculated fungi were probably greater than their cellulose decomposing capacity. However, maximum growth of all four inoculated fungi, i.e. A.fumigatus, Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus, were recorded at the pH 7.5 from both xylan and cellulose-containing agars. This has suggested that although the inoculated fungi were able to grow even in highly alkaline pH conditions (i.e. 9.5 in this experiment), their maximum growth could be more accurately determined at lower alkaline pH levels. It might plausibly be argued that the suppressed growth on agar at high pH levels was alternatively due to the effects of buffering system (Doskacil 1948). Nevertheless, no attempt was made to investigate the influence of the buffering system on the growth rates of the isolated fungi.

In general, however, it seemed that all four fungi used in this experiment were quite capable of growing under highly alkaline pH levels when xylan or cellulose were used as the sole carbon sources in the growth medium.

5.6. Discussion

In the above outlined study previously isolated fungi from barley straw (variety stores in the B.I.C. premises) were tested for their ability to utilize different carbon sources in the growth medium; while gorwth conditions were provided under the aklaline pH ranges.

The most active fungi found to be utilizing a varity of carbon sources were: Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus, which showed their strong clearing abilities and vigorous growth on agars and liquid media. All these fungi showed these characteristics under alkaline pH conditions and at a fixed incubation temperature, i.e. 30°C.

Thirty fungal species previously isolated from barley straw were tested for their ability to grow on 1% ball-milled cellulose agar medium. It may be concluded from the summarised results that most of the inoculated fungi showed vigorous growth, but failed to show good clearing on cellulose agar. This suggests that when cellulose was present in the medium, it was not acting as the sole carbon source, as there might be the possibility of utilization of other carbon components present in the growth medium such as L-Asparagine and yeast extract. The fungi showing those characteristics were Alternaria tenuis, Aspergillus flavus, A. versicolor, Aspergillus sp., Cephalosporium sp., Botrytis cineria, Fusarium spp., Gliocladium roseum, Humicola sp., Myriococcum albomyces, Paecilomyces sp., Scopulariopsis brevicaulis, Stachybotrys atra and Streptomyces sp. Some of the above-named fungi were previously reported by many workers as being cellulolytic, e.g. the species of Cephalosporium was

reported by Franz and Loub (1959) and Domsch and Gams as the one causing the degradation of textile and carboxymethyl cellulose (CMC). Domsch and Gams also reported the cellulolytic activities of Fusarium spp., Gliocladium sp and Humicola sp. Three species of Fusarium were also reported by Forbes and Dickinson (1977) as being cellulolytic after characterising these species by growing them over a range of temperatures pH levels supplementing them with different nitrogen sources in their growth media.

Fergus (1969) reported that Myriococcum albomyces showed greater cellulolytic activity at higher temperatures. He examined the degradation of filter paper and utilization of soluble C.M.C. by this fungus when incubated at higher temperatures. He also reported the degration of filter paper by some of the species of Streptomyces sp. Decomposition of wood by Stachybotrytis was tentatively reported by White, Mandels and Siu (1950); cellulose decomposition in the form of filter paper (Domsch, 1960) textile fibres (Borut, 1960) and methyl cellulose (Kanerskayo, 1960) have also been reported for this fungus.

Some of the fungi showed considerably less growth and definite, weak or negligible clearing of cellulose. This suggests that there might be the possibility of being unable to show the above characteristics under alkaline pH conditions. As was already mentioned, the medium used (i.e. Eggins and Pugh's cellulose agar) was also added with other carbon-containing components and those fungi concerned seemed to be Aureobasidium pullulans,

Cladosporium sp., Mucor sp., Penicillium spp. Rhizopus sp. Scopulariopsis sp. and S. brevicaulis. Han, Peter, Anderson, and Lekprayoon (1976) reported luxuriant growth of Aureobasidium pullulans on acid hydrolysate of rye grass straw. In addition, Domsch and Gams (1969) reported the most active enzymatic activity of Aureobasidium bolleyi as well as a few species of Penicillium. The C.M.C. decomposition rates which were being tested and negligible cellulolytic activity was reported for Botrytis cineria by these authors.

The fungi showing very strong cellulolytic activity and luxuriant growth on cellulose agar were found to be: Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus. The first-named fungus was isolated from cellulose film previously buried in soil (Daniels, 1961). Its isolation from cellulose-containing substrates was reported by many previous investigators, e.g. Tribe (1957, 1960) reported its isolation from buried strips of cellophane from soil ranging from neutral to alkaline pH. It should be borne in mind that this fungus can grow above pH 8.8 (Jensen, 1931). Good decomposition of C.M.C. by Botryotrichum piluliferum was reported by Domsch and Gams (1960) and by Jensen, 1931). Good cellulose decomposition from colonised straw was observed by Haider and Domsch (1969) for this fungus. Very strong cellulose decomposing abilities for Chaetomium globosum and Coprinus cinereus have been reported by several investigators, including Rege, 1927; Fries, 1955; Eastwood, 1952 and Chang, 1967).

The results for the xylanolytic activities of fungi lead to the conclusion that a surprisingly large number of

inoculated fungi possess the ability to decompose xylan more easily and more quickly than cellulose. Most of the fungi failed to show good clearing of cellulose agar, but showed strong or very strong activity where xylan was used. Flannigan (1970) reported the decomposition of arabinoxylan and carboxymethyl cellulose (C.M.C.). He used the fungal isolates which he had isolated from barley kernels. He found that Botrytis cineria, Aspergillus flavus and two species of Alternaria tenuis showed very strong xylanolytic activity and similar results have also been obtained in the work presented in this experiment. Domsch and Gams (1969) have also reported the decomposition of xylan by some soil fungi; they have also found the high xylanolytic activities of all the above-mentioned fungi, including Aspergillus versicolor; a similar characteristic was also shown by this fungus in the present experiment.

The early investigations of Waksman and Diehn (1930) have also shown the decomposition of hemicellulose by micro-organisms particularly by fungi. They found that the fungi Aspergillus fumigatus, Humicola sp., Mucor sp., Rhizopus sp., Botrytis cineria, Cladosporium and Cunninghamella sp. and Penicillium spp. were able to attack the hemicellulose of that endosperm of dates. A moderate clearing activity on xylan was reported here for the fungal species Mucor and Cladosporium, and a weak activity for Rhizopus and Penicillium, but the activities of Botrytis cineria and some other fungi listed in Table 19 verify the results of Waksman and Diehn.

Aureobasidium pullulans, Cephalosporium sp., Scopulariopsis brevicaulis, Scopulariopsis chartarum,

Scopulariopsis sp. and *Streptomyces* sp., on the other hand, have shown moderate to weak activities here. However, all the above named fungi, with the exception of *Cephalosporium* sp. and *Streptomyces* sp. were reported by Domsch and Gams (1969) as showing the most active xylanolytic activites. Flannigan (1969) also reported the weak acticities of *Aureobasidium pullulans* and *Cephalosporium* sp.

Botryotrichum piluliferum, *Chaetomium globosum* and *Coprinus cinereus* showed very strong xylanolytic activity clearing nearly the entire surface of the agar plates added with xylan (1% w/v). Their xylanolytic activities have also been reported by many previous investigators such as Rege, 1927; Jensen, 1931; Sorensen, 1953; Fries, 1955; Chang, 1967.

Fungus Aspergillus sp., *Botryotrichum piluliferum*, *Chaetomium globosum*, *Chaetomium* sp., *Penicillium* sp. and *Stachybotrys atra* were tentatively identified as Bavendamms polyphenoloxidase positive strains on the basis of the appearance of wide darkened zones around the mycelial mats of these fungi when growing on malt agar supplemented with 0.5% gallic or tannic acid. Actually, the Bavendamms test was a criterion for indicating the ligninolytic activities of fungi. However, various researchers have further substantiated the investigation of Bavendamm for the determination of ligninolytic activities of fungi, particularly wood-rotting (Preston and McLennan, 1948); Lindeberg, 1948; Etheridge, 1957; Nobles, 1958 and Gilbertson, 1975).

Enzymes play very important parts in the decomposition of lignin but the nature of the enzymes responsible for

polyphenoloxidase reactions has remained a matter of debate for many workers. Nevertheless, Fahraeus (1952) suggested that the enxymes secreted by the ligninolytic fungi were of the laccase type rather than of tyrosinase. Moreover, Henderson (1963) claimed that the mechanism of lignin decomposition was a matter of controversy among many workers involved in this type of research. All the literature hitherto available in this field provided an inadequate account of the complexities of phenolic structure. However, previous investigators reported that a number of soil fungi were capable of utilizing simple lignin-related phenolic compounds as the sole carbon sources; the reduction in methoxyl content of delignified materials in soil was also considered as an indication of the lignin decomposing abilities of soil fungi. (Sowden and Atkinson, 1949; Henderson and Farmer, 1955; Henderson, 1956).

Different types of lignins were used in the present work as the sole carbon sources in the growth media of isolated fungi, and the results given in Tables 24 and 25 showed the most active growth was revealed by Botryotrichum piluliferum, Chaetomium globosum and Humicola sp. All these fungi covered nearly the entire surface area of the agar plates.

Approximately half or more than half of surface of agar was covered by Gliocladium roseum, Myriococcum albomyces, Scopulariopsis brevicaulis, Stachybotrytes atra and Streptomyces sp. after the completion of the required incubation period. Nevertheless, comparing the rates of growth on 1% indulin A(R) and lignin prepared by 72% H_2Sc4 method, extensive radial growths of the above-mentioned fungi

were recorded from the agar medium supplemented with 1% lignosulphonate.

Similar results were also recorded with liquid cellulose inoculations, but the difference was noted from shake and stand culture incubation. More pelleted growth was viewed from some of the submerged cultures where shake culture incubation was used.

Some of the above outlined fungi were also reported by previous workers as ligninolytic, e.g. Haider and Domsch (1969) reported that Botryotrichum piluliferum was capable of decomposing a good proportion of lignin when colonising on straw. Chaetomium globosum being soft-rot was reported to be causing the decomposition of lignin content from spruce wood when the material was inoculated with this fungus under laboratory conditions by Levi (1965). Nevertheless, according to Ledingham (1942) the utilization of lignosulphonate was low with this fungus; in contrast, good growth was recorded in this work when Chaetomium globosum was inoculated under such conditions. Colonisation of wood led to losses in weight and stability; this was reported by Haider et al (1969) and Merrill, French and Hossfeld (1965), for some species of Humicola. Domsch (1960) reported the decomposition of lignonsulphonate by Scopulariopsis brevicaulis, while Duncan and Eslyn (1966) reported the soft-rot decay in timber of Stachytotrys atra.

Four different fungi, i.e. A. fumigatus, Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus, were further tested for growth pattern by using 1% xylan or cellulose as the sole carbon source. When the agar media were adjusted to a range of alkaline pH levels 7.5, 8.5 and 9.5. However, the results have already indicated that,

although the above inoculated fungi have shown their growth on the media adjusted at pH 9.5, the best growth was evaluated at pH 7.5 which both xylan agar and cellulose agar media. This indicates that these fungi were quite capable of utilizing the complex carbon sources even under highly alkaline pH conditions. The work presented in the next chapter will further substantiate the growth activities of the above named fungi and some other selected isolates; colonisation on barley straw will be evaluated under alkaline conditions. This was supposed to be one of the most important parts of this investigation which examines biological up-grading of straw by means of fungi which can utilize complex carbon sources under highly alkaline pH conditions.

CHAPTER 6

Colonisation of barley straw by selected fungi under sterile and non-sterile conditions

CHAPTER 6

6.1. INTRODUCTION

As was mentioned in the introductory chapter of this thesis, the work described here was a part of investigation into the development of a semi-solid fermentation technique for converting straw into a ruminant feed.

Previous B.I.C. workers involved in this research area had selected Coprinus cinereus as the main agent for up-grading straw. This was chosen because this fungal species is highly cellulolytic and can dominantly colonise straw under a wide range of pH and temperature conditions. Moreover, there were less chances of competition with other fungi when non-sterile techniques were involved for the up-grading of straw. (Seal, 1973; McShane, 1976).

After consideration of the investigation outlined above, this part of the work was designed to investigate the colonisation of barley straw by selected fungi under alkaline pH conditions. The fungi used were previously isolated from barley straw under the above-mentioned pH conditions and at a wide range of incubation temperatures (see Chapters 2 and 4).

For obtaining a range of alkaline pH levels, 1% and 0.5% V/V aqueous ammonia was added separately into mineral salt medium. This also provided the sole nitrogen source in the medium. However, in addition, another nitrogen source, i.e. L.asparagine, was used in another set of mineral salt medium and the required pH levels were obtained by the use of buffering systems (Lindeberg, 1939, 1944). This was

done to investigate the difference in the rates of fungal colonisation and pH change when straw was soaked separately in two different liquid media supplemented with the two previously mentioned nitrogen sources.

Apart from the utilization of two described nitrogen sources, no attempt was made to study the effect of various other nitrogen sources on the rates of decomposition of straw. However, it is assumed on the basis of the findings of previous researchers, that the relative combination of carbon and nitrogen play a key role in the growth and breakdown of plant materials by the action of micro-organisms. The early investigation of Woling (Rege, 1927) showed that amounts of nitrogen contained in organic materials controlled the rate of decomposition. Rahn (Rege, 1927) suggested that the application of nitrate or ammonical nitrogen accelterated the decomposition of straw. This point was further confirmed by Hutchinson and Richards (Rege, 1927) when they first discovered the quantitative combination of nitrogen and carbohydrate. They reported further that nitrogen thus applied in a soluble condition was temporarily immobilised, and that the amount of nitrogen the straw was thus capable of locking-up was equal to that necessary for prounounced rotting. A conflicting results was reported by Richards and Amoore (Rege, 1927) who found that even after the addition of assimible nitrogen, certain organic materials such as sawdust, rice husk, old broken or coconut shell cannot be decomposed. On the other hand, certain others like banana stem or hop-bines require a long period for deomposition.

The investigations of the above-named workers suggested that a supply of easily assimilated nitrogen compounds in

suitable concentrations could cause the rapid breakdown of straw by providing aerobic and neutral or slightly alkaline conditions suitable for growth. Levi and Cowling (1968) also found that the breakdown of ligno-cellulose materials increase with the addition of nitrogen sources. In addition to that, Morton and McMillan (1954) studied the application of different nitrogen sources in growth medium of fungi and concluded their report by suggesting that ammonia ion was a good organic source of nitrogen for the growth of a large number of fungi.

Literature from previous work showed that aqueous ammonia was used by workers to increase the digestibility of straw but, apart from the work being carried out at the B.I.C., no attempt had been made to biologically up-grade ammonia impregnated straw. In this work, one of the purposes of using this chemical was to provide conditions under which the colonised fungi could utilize the nitrogen of the ammonia molecule and increase the protein content by producting their mycelial growth.

6.2. EXPERIMENTAL PROCEDURE

Two separate sets of experiments were carried out to investigate the colonisation of barley straw by selected fungi under alkaline pH conditions. In the first set aseptic technique was employed; the second set however, were conducted under non-sterile conditions throughout the experiments. This was done to determine the probability of the growth of un-inoculated fungi during substrate colonisation with an added inoculum.

Two different nitrogen sources were used, aqueous ammonia and L.asparagine. The advantage of using aqueous ammonia was that there was no need to adjust the pH level of the growth medium to obtain an alkaline pH range, whereas a buffer system was required to produce an alkaline pH level when L.asparagine was used.

The straw used in this experimental work was from the same bale as the one previously used in the isolation programme. The straw was cut into lengths of 4-6 cm by means of a guillotine.

Two separate sets of mineral salt solutions were prepared without any addition of carbon and nitrogen sources. 1% V/V aqueous ammonia was added to half of the first solution, and 0.5% to the remainder. With the second solution 1 gramme W/V L.asparagine was added per litre of liquid medium, and the pH was adjusted to an alkaline pH level by the use of sorensen phosphate buffer solutions (Lingeberg, 1939, 1944).

6.2.1. Colonisation under sterile conditions

Five grammes by weight chopped pieces of straw were placed in 250 ml erlenmeyer conical flasks and a small quantity of distilled water was added to each flask to facilitate the process of sterilization. After covering the mouth of the flasks loosely with non-absorbent cotton-wool bungs, the contents were autoclaved at 15 lbs p.s.i for 20 minutes at 121°C. After autoclaving, the flasks were arranged in two sets. The first set was further divided into two separate portions, and each was marked for 1 or 0.5% ammonia concentrated mineral salt solution. All flasks in the second set were marked for L.asparagine supplemented solutions (All sets of flasks containing equal amounts of straw, i.e. 5 grammes in each flask) were added with 20 ml aliquots of their respective mineral salt solutions which were previously autoclaved. To soak the straw with the added liquid medium the solutions were sprayed gently using a sterile 20 ml syringe onto straw in the flasks to provide the conditions for semisolid culture after colonisation with fungal isolates. Additionally, each flask was vigorously agitated after the non-absorbent cotton wool had been replaced to seal the mouth. This was done to re-distribute the remaining aliquots which had collected at the base of the flasks after impregnation with the liquid medium. After ensuring that the straw in the slasks was adequately impregnated, 13 isolated fungi were separately inoculated by placing in the flasks 6 mm mycelial plugs cut from the edges of the agar stock cultures and which had been maintained on potato dextrose agar for one week prior to this experiment. Five replicates were

prepared for each inoculum and all the flasks were inoculated at 30°C for 10 days.

6.2.2. Colonisation under non-sterile conditions

Similar procedures were employed in this part, to those reported in section 6.2.1., except that the straw content and mineral salt solutions were not autoclaved. Nevertheless, non-absorbent cotton-wool bungs were applied to cover the mouth of each flask. This was done to control the evaporation of the added solutions from flasks rather than to control against possible contamination as the whole experiment was run under non-sterile conditions.

6.2.3. Assessment of fungal growth rates and pH measurements after incubation

Growth rates were estimated by evaluating the substrate coverage of colonised fungus and were recorded in terms of - or + signs.

A negative sign (-) indicated that fungal growth was not visible macroscopically, while the positive (+) sign denoted negligible growth. Also used were ++; and +++ and ++++, where ++ represented coverage of slightly less than half the substrate area; +++ more than half and finally ++++ indicates that the entire area was covered. After fungal growth had taken place in each flask, the final pH of the remaining liquid medium was measured using a Pye Model 78pH meter.

6.3. RESULTS

6.3.1. Colonisation under sterile conditions

Tables 27 and 28 summarised the results for fungal colonisation on barley straw. Each figure illustrates the average from 5 replicate flasks. (Plates 3a, b and c also indicate fungal colonisation on mineral salt amended medium after 5 days incubation at 30°C.)

Table 27 indicates that the most rapid fungal colonisation of straw was shown by *Botryotrichum piluliferum*, *Coprinus cinereus*, *Humicola* sp., *Myriococcum albomyces* and *Scopulariopsis brevicaulis* from liquid media supplemented with both 1% and 0.5% aqueous ammonia. The final pH reading obtained from the residual liquid media of these revealed that the fungi were still growing under alkaline pH conditions even after a 10 day incubation period. It is also apparent from Table 28 that these fungi also produced similar results where the liquid medium was supplemented with L.asparagine. A similar result was also obtained for *Chaetomium globosum* and *Gliocladium roseum* from both types of nitrogen supplemented media. Nevertheless, these fungi showed rather less mycelial extension when colonising on straw treated with a liquid medium containing 1% ammonia. It is also important to mention here that during the first few days of the incubation period (from about the first, second or third day) flasks were viewed macroscopically, and most of the above named fungi showed no signs of development of radial growth. Some of the inoculated fungi failed to colonise or showed negligible growth on ammonia-containing media. On the other hand they showed extensive growth when L.asparagine was added to the growth medium. These fungi

Table 27. Colonisation of selected fugni on barley straw treated with mineral salt solution containing 1 or 0.5% NH_3, after 10 days incubation at 30°C (under sterile conditions)

Fungi	Mycelial growth on solution containing 1% NH_3 with pH 10.6	Final pH level	Mycelial growth on solution containing 0.5% NH_3 with pH 10.2	Final pH level
Aspergillus fumigatus	+	8.50	++	7.80
A.versicolor	+	8.00	++	7.15
Alternaria tenuis	-	8.20	+	6.80
Botryotrichum piluliferum	++++	8.62	++++	8.90
Chaetomium globosum	++	8.76	++++	8.35
Coprinus cinereus	++++	7.77	++++	7.73
Gliocladium roseum	+++	8.26	++++	7.72
Fusarium sp.1	-	8.97	+	8.90
Humicola sp.	++++	8.16	++++	6.71
Myriococcum albomyces	++++	8.80	++++	7.76
Scopulariopsis brevicaulis	++++	9.05	++++	8.10
Stachybotrys atra	-	8.76	-	8.68
Streptomyces sp.	+	9.56	++	8.41

++++ = Extensive mecelial growth covering entire surface area of the straw

+++ = Moderate mecelial growth covering more than half of the surface area

++ = Fair mycelial growth covering slightly less than half of the surface

+ = Negligible mecelial growth

- = No growth was apparent

Table 28. Colonisation of selected fungi on barley straw soaked in mineral salt supplemented with ammonium sulphonate as external nitrogen source, after incubation at 30°C for 10 davs under sterile conditions of growth (pH 8.2)

Fungi	Measurement of mycelial growth	Final pH level
Alternaria tenuis	+++	7.28
Aspergillus fumigatus	+++	8.20
A.versicolor	++++	7.60
Botryotrichum piluliferum	++++	7.16
Chaetomium globosum	++++	7.55
Coprinus cinereus	++++	8.09
Cliocladium roseum	++++	7.92
Fusarium sp.1	++	7.50
Humicola sp	++++	7.65
Myriococcum albomyces	++++	8.23
Scopulariopsis brevicaulis	++++	8.11
Stachybotrys atra	++++	7.29
Streptomyces sp.	++	8.40

++++ = Extensive mycelial growth covering entire surface area of the straw

+++ = Moderate mycelial growth covering more than half of the surface area

++ = Fair mycelial growth covering slightly less than half of the surface area

+ = Negligible mycelial growth

- = No growth was apparent.

Colonisation of fungi on barley straw amended with basal liquid medium (buffered to pH 8.20), after five days incubation at 30^{0}C.

Plate 3 a *

Plate 3 b **

Plate 3 a *

13 = Chaetomium globosum

3 = Aspergillus fumigatus

4 = Streptomyces sp

51 = Gliocladium roseum

49 = Aspergillus sp.

Plate 3 b **

7 =Stachybotrys atra

11=Myriococcum albomyces

5 =Fusarium sp.

8 =Scopulariopsis brevicaulis

Plate 3 c ***

Plate 3 c ***

2 = *Aspergillus versicolor*

10 = *Aureobasidium pullulans*

14 = *Alternaria tenuis*

16 = *Botryotrichum piluliferum*

were: Alternaria tenuis, Aspergillus fumigatus, A. versicolor, Fusarium sp. and Stachybotrys atra. Their growth medium also revealed neutral or slightly alkaline pH ranges.

6.3.2. Colonisation under non-sterile conditions

Results are summarised in Tables 29, 30 and 31 for fungal colonisation on straw. The tables indicate that some other fungi were also seen growing during straw colonisation with a particular inoculum. Nevertheless, the appearance and rates of colonisation of uninoculated fungi were mostly dependent either on the presence of added aqueous ammonia in the media or the types of fungal inoculum used for colonisation. It is important to note that, throughout the incubation period the uninoculated fungi appeared to be those which were seen to be abundantly isolated from the straw used in previous investigations on fungal isolation (see chapters 2 and 4).

These fungi were Aspergillus fumigatus, Aspergillus sp Chaetomium globosum, Chaetomium sp., Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp. On the basis of some characteristics possessed by some of these fungi they were very difficult to identify up to genera level when macroscopic and microscopic examination was made. However, random samples from the flasks were inoculated onto agar plates for further identification by examining the presence of white cottony mycelium and the appearance of conidiophores or the short fluffy fruiting initials for Coprinus species. Whereas the appearance of perithecia was examined for the isolation of Chaetomium species.

Extensive colonisation of straw was shown by the inoculated fungi: Botryotrichum piluliferum, Coprinus cinereus,

Table 29. Colonisation of selected fungi on barley straw treated with mineral salt solution containing 1% NH_3, after 10 days incubation. (Under non-sterile conditions of growth.)

Fungi	Mycelial growth on solution containing 1% NH_3 added solution	Appearance of other fungi	Final pH level of medium
Alternaria tenuis	-	Coprinus sp Chaetomium sp Scopularcopsis	8.20
Aspergillus fumigatus	++	Coprinus sp Chaetomium sp	8.00
A.versicolor	+	Coprinus sp Chaetomium sp	7.80
Botryotrichum piluliferum	++++	-	7.8
Chaetomium globosum	+++	Coprinus sp Scopulariopsis sp	7.56
Coprinus cinereus	++++	-	7.30
Gliocladium roseum	++++	-	7.30
Fusarium sp.l.	-	Coprinus sp Chaetomium sp	7.70
Humicola sp.	+++	Chaetomium sp	7.15
Myriococcum albomyces	+++	Aspergillus sp Scopulariopsis sp	7.48
Scopularipsis brevicaulis	+++	-	7.87
Stachybotrys atra	-	Coprinus sp Chaetomium sp	
Streptomyces sp	+	Scopulariopsis sp	8.45

++++ = Extensive mycelial growth covering entire surface area of the straw
+++ = Moderate mycelial growth covering more than half of the surface
++ = Fair mycelial growth covering slightly less than half of the surface
+ = Negligible mycelial growth
- = No growth was apparent

Table 30. Colonisation of selected fungi upon barley straw treated with mineral salt solution containing 0.5% NH_3 after 10 davs of incubation at 30°C. (Under non-sterile conditions of growth)

Fungi	Mycelial growth on solution containing 0.5% NH_3	Appearance of other fungi	Final pH level of medium
Alternaria tenuis	-	Coprinus sp Chaetomium sp	7.35
Aspergillus fumigatus	++	Coprinus sp Chaetomium sp Scopulariopsis sp	7.15
A. versicolor	++	Coprinus sp Chaetomium sp Scopulariopsis sp	7.77
Botryotrichum piluliferum	++++	-	7.30
Chaetomium globosum	++++	-	7.25
Coprinus cinereus	++++	-	7.15
Gliocladium roseum	+++	Chaetomium sp Coprinus sp Aspergillus sp	7.40
Fusarium sp.1	-	Chaetomium sp Coprinus sp Aspergillus sp Scopulariopsis sp	7.11
Humicola sp	+++	Chaetomium sp Streptomyces sp	7.60
Myriococcum albomyces	++++	Chaetomium sp Scopulariopsis sp	7.30
Scopulariopsis brevicaulis	++++	Streptomyces sp	8.10
Stachybotrys atra	-	Aspergillus sp Coprinus sp	7.60
Streptomyces sp	++	Botrytis sp Scopulariopsis sp	7.56

++++=Extensive mycelial growth covering entire surface area of the straw
+++ =Moderate mycelial growth covering more than half of the surface area
++ = Fair mycelial growth covering slightly less than half of the surface area
\+ = Negligible mycelial growth
\- = No growth was apparent

Table 31. Colonisation of selected fungi on barley straw soaked in mineral salt solution supplemented with ammonium sulphonate as the external nitrogen source, after incubation at 30°C for 10 days under non-sterile conditions of growth (pH:8.2).

Fungi	Measurement of mycelial growth	Appearance of other fungi	Final pH level of medium
Alternaria tenuis	+	Aspergillus sp Chaetomium sp	7.8I
Aspergillus fumigatus	++	Aspergillus sp Chaetomium sp Coprinus sp	7.88
A. versicolor	+	Aspergillus sp Chaetomium sp Coprinus sp Scopulariopsis sp	6.65
Botryotrichum piluliferum	++++	Aspergillus sp	7.96
Chaetomium globosum	++++	Coprinus sp Scopulariopsis sp	7.70
Gliocladium roseum	++++	Aspergillus sp Chaetomium sp Coprinus sp	7.55
Fusarium sp.1	+	Aspergillus sp Chaetomium sp Coprinus sp Scopulariopsis sp	7.80
Coprinus cinereus	++++	Aspergillus sp Chaetomium sp	7.96
Scopulariopsis brevicaulis	++++	Aspergillus sp Chaetomium sp	8.59
Stachybotrys atra	+	Aspergillus sp Chaetomium sp Coprinus sp	6.25
Streptomyces sp	+	Aspergillus sp Chaetomium sp Coprinus sp	7.84

++++ = Extensive mycelial growth covering entire surface area of the straw
+++ = Moderate mycelial growth covering more than half of the surface area
++ = Fair mycelial growth covering slightly less than half of the surface area
\+ = Negligible mycelial growth
\- = No growth was apparent

Gliocladium roseum and Scopulariopsis brevicaulis when flasks were supplemented with liquid media containing 1% aqueous ammonia. It is important to mention here that the appearance of other fungi was not detected either macroscopically nor by examining microscopically the random samples taken from the flasks. This indicates their dominant colonizing abilities upon this organic substrate (i.e. straw) under the influence of highly alkaline pH conditions. The figures in table 27 show that even after the extensive growth of these above-named fungi, liquid media measurement still revealed alkaline pH ranges. Results similar to those for 1% ammonia were obtained for the above-named fungi when the flasks with liquid containing 0.5% aqueous ammonia were viewed and the medium left after growth tested to determine their final pH level. However, the presence of un-inoculated Streptomyces sp was noticed in the flask containing straw initially inoculated with the fungus Scopulariopsis brevicaulis. Chaetomium globosum and Gliocladium roseum and Myriococcum albomyces also showed rapid colonisation on straw treated with liquid media supplemented with either 1% or 0.5% aqueous ammonia or with L. asparagine (0.1%), but the appearance of other fungi such as Aspergillus sp., Chaetomium globosum, Chaetomium sp., Coprinus cinereus and Scopulariopsis brevicaulis was also noticed. Very similar results were shown by the above-named inoculated fungi in the flasks supplemented with L. asparagine but comparatively (i.e. from flask marked for 1% or 0,5% aqueous ammonia) thick mycelial growth was observed under those conditions. The final pH measurement also showed neutral or slightly alkaline pH range.

It was somewhat difficult to evaluate the colonisation rates when the fungi Alternaria tenuis, Aspergillus versicolor, A. fumigatus, Fusarium sp., Stachybotrys atra and Streptomyces sp were inoculated either on the straw treated liquid media containing 1% or 0.5% ammonia or supplemented with L. asparagine. Most of the un-inoculated fungi, e.g. Aspergillus sp, Chaetomium sp. Coprinus sp and Scopulariopsis sp were seen covering most of the surface area of straw. Nevertheless random straw samples were viewed on illuminated microscope at high magnification and also were inoculated on agar plates to subculture them for further identification.

The above-mentioned method evaluated their presence and on that basis it is assumed that the presence of these inoculated fungi had gradually decreased with the increasing rates of substrate colonization by uninoculated fungi (e.g. the rates of colonisation for those fungi under sterile conditions. See tables 28 and 29).

6.4. INFLUENCE OF HIGHER INCUBATION TEMPERATURE AND THE RATE OF COLONISATION OF SOME SLECTED THEROMOTOLERANT FUNGI ON BARLEY STRAW

6.4.1. Experimental procedure

Four thermotolerant fungi, Aspergillus fumigatus, Botryotrichum piluliferum, Coprinus cinereus and Myriococcum albomyces, were selected to examine their colonisation on barley straw under sterile and non-sterile conditions at a range of higher incubation temperatures. The method used for inoculation onto straw treated with 1% aqueous ammonia similar to that previously described in section 6.2.1. All four inoculated fungi were incubated at 35°C, 40°C, 45°C and 50°C for ten days; five replicates were made for each fungus.

6.5. RESULTS

Results are summarised in Tables 32A and 32B and in Figure 4, which show that under sterile growth conditions, the most suitable incubation temperature was 35°C, at which all the inoculated fungi showed maximum substrate colonisation. Nevertheless, the rate of colonsiation of A. fumigatus was considerably less in compariosn with the rest of the fungi at higher temperatures. After looking at the fungal colonisation on straw in relation to higher incubation temperatures under sterile conditions, one could see that A. fumigatus and Myriococcum albomyces continued to grow up to 50°C, but their rates of substrate colonisation were very low at that incubation temperature. The fungus Botryotrichum piluliferum, on the other hand, was able to colonise at temperatures up to 45°C, but the extent

Table 32. Influence of higher incubation temperatures on the colonisation of some selected fungi on barley straw (soaked in 1% aqueous ammonia supplemented mineral salt solution) after 10 daysincubation, A: under sterile conditions, and B: under non-sterile conditions.

A. (Sterile conditions)

Fungi	Fungal colonisation and pH measurement at different incubation temperatures							
	35°C	pH	40°C	pH	45°C	pH	50°C	pH
Aspergillus fumigatus	2 +	7.95	+	6.1	+	7.20	+	6.35
Botryotrichum piluliferum	4 +	8.50	2 +	7.11	+	6.25	-	5.88
Coprinus cinereus	4 +	8.00	2 +	7.40	-	6.55	-	6.30
Myriococcum albomyces	4 +	7.80	3 +	7.20	2 +	5.90	+	5.55

B. (Non-sterile conditions)

Fungi	35°C	pH	40°C	pH	45°C	pH	50°C	pH
Aspergillus fumigatus	2 +	7.00	+	6.80	+	6.55	+	5.80
Bortyotrichum piluliferum ***	4 +	8.10	2* +	7.60	+*	5.90	*** -	6.22
Coprinus cinereus	4 +	7.80	2* +	7.60	-*	5.80	*** -	5.50
Myriococcum albomyces	4 +	8.00	3 +	6.80	2* +	6.00	+*	5.80

+ = Growth positive but negligible

2
+ = Fair growth covering slightly less than half the surface area of the straw

3
+ = Moderate growth covering more than half the surface area of the straw

4
+ = Extensive growth covering almost the entire surface area of the straw

* = Presence of Aspergillus fumigatus was examined

**= Presence of Cephalosporium sp was examined

*** = Presence of unidentified bacteria and actinomyceters were noted

Influence of higher incubation temperatures on the colonisation of some thermotolerant fungi on barley straw

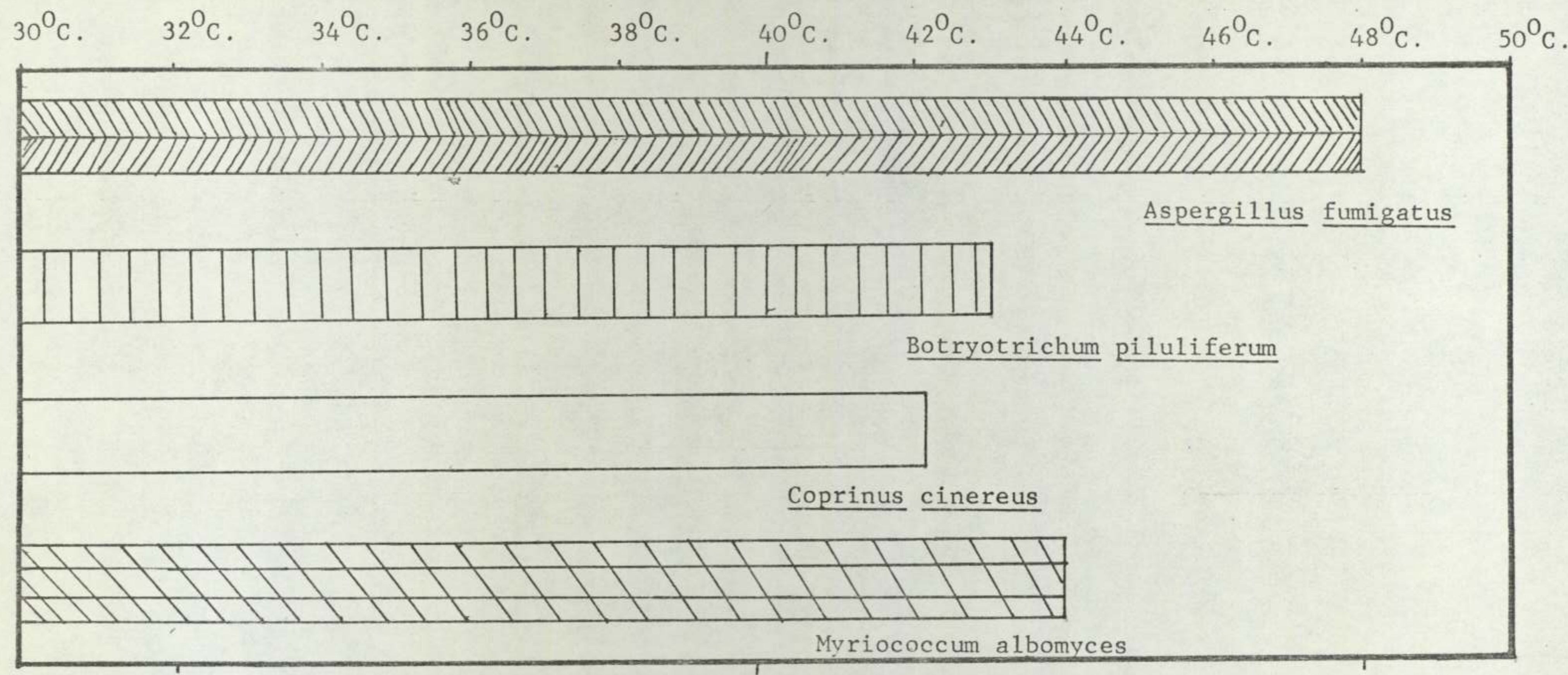


Figure: 4

of colonisation was also negligible at this temperature. Coprinus cinereus continued to colonise extensively up to 40°C, while, according to the investigation of Fries (1955), this fungus was able to grow up to 44°C incubation when she incorporated a growth factor, methionine, into the medium. McShane (1976) has virtually replicated the work of Fries, and has observed a similar result.

Table 32B illustrated similar results for non-sterile substrate colonisation by the above outlined fungi. Nevertheless, the appearance of the uninoculated fungi A. fumigatus and Cephalosporium sp were also seen from the flasks initially inoculated with fungal inocula. A.fumigatus was seen growing in the flasks initially inoculated with Botryotrichum piluliferum, Coprinus cinereus and Myriococcum albomyces up to the 50°C incubation temperature. Cephalosporium sp was seen appearing in the flasks initially inoculated with Botryotrichum piluliferum and C.cinereus, from the flasks incubated at 50°C. It is important to note that good substrate colonisation was shown by Myriococcum albomyces at 40°C, and no other contaminants were observed by either macroscopic or microscopic examination of the pieces of straw or even by plating out the random straw samples on agar plates. This fungus was previously reported by Cooney and Emerson (1944) to be capable of tolerating up to 57°C temperature and its maximum growth was found to be between 32-42°C.

A number of actinomycetes and bacteria were also viewed during the microscopic scanning of pieces of straw colonised by fungi, but no attempt was made to investigate the growth activities of these organisms in the present work.

pH measurement from the flasks under both sterile and non-sterile growth conditions showed that a gradual decrease in pH level was recorded for the inoculated fungi from the incubation temperature 35°C to 50°C. At, 35°C, most of the fungi showed a pH level within the alkaline range, whereas at 50°C acid pH levels recorded. The method of pH measurement was similar to that described earlier (6.2.1.).

6.6. SYNERGISTIC OR ANTAGONISTIC EFFECTS OF SOME SELECTED FUNGI DURING SUBSTRATE COLONISATION

Six cellulolytic or xylanolytic fungi were selected to investigate their synergistic or antagonistic effect,when growing togetheron agar or colonising straw under non-sterile conditions. For the aseptic technique, two types of fungi were inoculated together onto the agar plates, while the straw soaked in growth medium was placed in plastic boxes, and the inoculation made from fungi previously grown on potato dextrose agar by mixing fungus with straw.

6.6.1. Experimental procedure

A: Synergistic or antagonistic effectof fungi after inoculation on agar plate

Cultures were made of six different fungi, i.e. *Aspergillus fumigatus*, *Botryotrichum piluliferum*, *Chaetomium globosum*, *Coprinus cinereus*, *Gliocladium roseum* and *Myriococcum albomyces*, which maintained on potato dextrose agar for one week prior to the experiment. Malt agar was prepared (see appendix) and adjusted at the pH 7.7 by the aseptic addition of 1N sodium hydroxide up to the required

amount after 20 ml aliquots had been poured into sterile petridishes and the agar had solidified. Aseptic inoculation was made by placing two different 6mm fungal agar blocks at the centre of malt agar at a distance of 1 cm apart from each other. The agar blocks were cut from the edges of growing fungal mycelium on potato dextrose agar by means of a sharp, sterile 6 mm cork borer. Four replicates were prepared for each pair of fungi inoculated onto agar plates, and these were incubated at 30°C for 10 days.

6.6.2. B: Fungal role as dominant or suppressed coloniser on straw under non-sterile conditions

Adequate quantities of straw, previously soaked in mineral salt medium containing 1% V/V aqueous ammonia (see appendix) were placed in mushroom cultivating plastic boxes and the cultures from two different fungi were inoculated together onto the straw. The fungi used for inoculation had been grown on petridishes containing 20 ml aliquots of potato-dextrose agar one week prior to the experiment. The entire mycelial growths from different fungal agar plates were scraped off by means of a scalpel and thoroughly mixed with the straw. Six different fungi were used, and each fungus was inoculated individually with five other fungi. The boxes were incubated at 30°C for 10 days. They were covered with polythene sheeting to prevent any evaporation of moisture but not to control contaminations.

6.7. RESULTS

The results obtained with fungi grown together on agar plates or on straw are summarised respectively in tables 33 and 34. The inoculated fungi Botryotrichum piluliferum and Coprinus cinereus showed dominant growth when inoculated with other fungi onto the same agar. Nevertheless, no antagonistic effects were noticed with these fungi; other fungi showed their growth in comparison with these fungi. It was also observed that, when the above-named fungi were inoculated together, their growth rates were very similar. It was also noticed that during their radial expansion on the agar plates, their growth did not overlap. An example of an actively dominant fungus seems to be Chaetomium globosum, which suppressed the growth of A. fumigatus, Gliocladium roseum and Myriococcum albomyces, but its own growth was suppressed when inoculated with either Botryotrichum piluliferum or C. cinereus.

It was difficult to judge the antagonistic role with two fungi inoculated together onto straw. It was also difficult to verify the substrate colonisation rates from inoculated fungi. However, the dominant substrate colonisation of inoculated fungi could be deduced on the basis of macroscopic examination; on observing the colour of a particular growing mycelium; from the presence of perithecia or from some other relevant and distinguishing characteristics.

The results in table 34 show the most dominant colonisation for Botryotrichum piluliferum and Coprinus cinereus, when they were inoculated with other fungi to colonise straw. The growth of other fungi in comparison with these seemed to be suppressed but not inhibited as the

Table : 33 Synergistic or antagonistic effects of some isolated cellulolytic fungi after inoculating two isolates together onto agar plates (incubation 10 days at 30°C at pH 7.7

Fungi inoculated together		Sym-biosis	Antag-onism	Growth	Dominant fungus
Aspergillus fumigatus	Coprinus cinereus	+	-	++	Coprinus cinereus
A. fumigatus	Chaetomium globosum	+	-	++	Chaetomium globosum
A. fumigatus	Botryotrichum piluliferum	+	-	++	Botryotrichum piluliferum
A. fumigatus	Gliocladium roseum	+	-	++	Cunningham-ella elegans
A. fumigatus	Myriococcum albomyces	+	-	++	Myriococcum lbomyces
Botryotrichum piluliferum	Chaetomium globosum	+	-	++	Botryotrichum piluliferum
B. piluliferum	Coprinus cinereus	+	-	++	Both fungi
B. Piluliferum	Gliocladium roseum	+	-	++	B. piluliferum
B. Piluliferum	Myriococcum albomyces	+	-	++	B. piluliferum
Chaetomium globosum	Coprinus cinereus	+	-	++	C.cinereus
C. globosum	Gliocladium roseum	+	-	++	C.globosum
C. globosum	Myriococcum albomyces	+	-	++	C.globosum
C. cinereus	Gliocladium roseum	+	-	++	C.cinereus
C. Cinereus	Myriococcum albomyces	+	-	++	C.cinereus
Gliocladium roseum	Myriococcum albomyces	+	-	++	Both fungi

* = ++ indicate growth of both inoculated fungi on the agar plate.

Table 34. Fungal role as dominant colonisers after inoculating together onto straw (soaked in mineral salt solution), and incubated for ten days at 30°C at pH 7.7

Fungi inoculated together		Dominant fungus
Aspergillus fumigatus	Coprinus cinereus	Coprinus cinereus
A. fumigatus	Chaetomium globosum	Chaetomium globosum
A. fumigatus	Botryotrichum piluliferum	Botryotrichum piluliferum
A. fumigatus	Gliocladium roseum	Gliocladium roseum
A. fumigatus	Myriococcum albomyces	Myriococcum albomyces
Botryotrichum piluliferum	Chaetomium globosum	Botryotrichum piluliferum
B. piluliferum	Coprinus cinereus	Coprinus cinereus
B. piluliferum	Gliocladium roseum	Botryotrichum piluliferum
B. piluliferum	Myriococcum albomyces	Botryotrichum piluliferum
Chaetomium globosum	Coprinus cinereus	Coprinus cinereus
C. globosum	Gliocladium roseum	Chaetomium globosum
C. globosum	Myriococcum albomyces	Chaetomium globosum
Coprinus cinereus	Gliocladium roseum	Coprinus cinereus
C. cinereus	Myriococcum albomyces	Coprinus cinereus
Gliocladium roseum	Myriococcum albomyces	Gliocladium roseum

random straw samples from pieces of colonising straw were viewed microscopically, and the presence of other inoculated fungi was ascertained. The presence of other incoulated fungi was also noted on microscopic examination, particularly the abundant appearance of A. fumigatus. Nevertheless, no attempt was made to properly identify those fungi as their growth rates were considerably lower.

Apart from dominant colonisation of Coprinus cinereus and Botryotrichum pilulferum (reported to be the imperfect stage of C. globosum, by Daniels, J.1961), the fungus Chaetomium globosum was found to be dominant, as it produced extensive growth and perithecia when inoculated with other fungi.

CHAPTER 7

GENERAL DISCUSSION AND SUGGESTIONS

7. GENERAL DISCUSSION

The work presented in this thesis is an attempt at the biological up-grading of barley straw in the hope of producing a potentially valuable end product which would be palatable to ruminant livestock. The steps involved the isolation of fungi from barley straw under alkaline pH conditions, and at a range of incubation temperatures. An assessment was made of the utilization of various carbon sources from the common isolated fungi and their colonisation rates on straw were also studied under sterile and non-sterile conditions. On the basis of this, the isolated fungi were characterized and the fungal selection was made for choosing specific fungi to use as the agents for the biological up-grading of straw, and to enhance its value as a foodstuff of this potentially important agricultural by-product.

Chapter 2 shows the isolation of mesophilic fungi from barley straw using four different isolation techniques; direct inoculation, inoculation of washed straw, inoculation of washings from straw and the inoculation of incubated straw treated with aqueous ammonia. Comparison of the results of all these techniques showed that the largest number of fungi were isolated when the direct inoculation method had been used. The disadvantage in this technique is that no distinction can be made between fungi present on the straw surface as spore contaminants and fungi whose spores have germinated and penetrated into the epidermis On the other hand, this technique permitted the study of the isolation of the largest number of fungi which were established on the straw or present as surface propagules - at the time of inoculation - and which appeared under the influence of

alkaline pH levels on growth media.

When the washing technique was employed for the isolation of the dominant colonisers on straw, the number of fungi isolated was considerably less than the total of those isolated by the direct inoculation method. This shows that most of the surface propagules found on the straw were washed out during the successive washings and before the inoculation of the straw pieces onto agar media.

The difference in the isolation of fungi from both the direct inoculation and inoculation of washed straw was noticed by inoculating samples of the water from successive washings on agar media. Most of the fungi which appeard on agar media by this method were the ones isolated by direct inoculation. In other words, most of the surface propagules which were washed out during successive washings of straw were isolated on the agar plates.

In another method of isolation, the straw was previously soaked in 1% v/v aqueous ammonia added mineral salts media and incubated for a period of time. Then samples from the straw pieces were examined microscopically and a number of fungi were viewed. When these samples were inoculated onto cellulose agar plates most of the microscopically examined fungi were isolated. This was also reported from direct inoculation and inoculation of washed straw pieces by using a range of aqueous ammonia added mineral salt media. Nevertheless, some of the fungi including *Aspergillus flavus*, *A. versicolor*, *Aureobasidium pullulans*, *Botryotrichum pululiferum* and *Penicillium* spp. which were not isolated from the above mentioned isolation conditions also appeared on cellulose agar.

Two sets of agar media were used for fungal isolation. The first set consisted of mineral salt agar supplemented with a range of aqueous ammonia concentrations, i.e. 1%, 0.5, 0.25, 0.125 and 0.625; while the second set consisted of cornmeal agar, czapex dox agar, potato dextrose agar, cellulose-glucose agar, and cellulose-starch agar. The purpose of using the first set of media was twofold: firstly, to facilitate the growth conditions for the isolation of only those fungi which could possibly utilize the inoculated straw itself as the sole carbon source by decomposing its various carbonaceous components. Secondly, to provide the nitrogenous source for the isolated fungi and to adjust the pH's of the media under the alkaline pH level without the usage of a buffering system. This shows the chances for isolating only those fungi which were able to decompose straw as their carbon source and also utilize aqueous ammonia as the nitrogen source. In addition, those able to tolerate the presence of the aqueous ammonia in their growth media and also possessing the ability to grow under the highly alkaline pH conditions produced by the addition of this organic chemical were also isolated. It was noticed from preliminary investigations that in addition to the role mentioned above, the ammonia solution also acted as the strong buffering agent which maintained the stability of the pH level for a prolonged period.

The most common fungi isolated from the above outlined growth conditions were tentatively identified as Aspergillus fumigatus, Aspergillus sp., Chaetomium globosum, Chaetomium sp., Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp. Most of these isolates were found to be highly cellulolytic

and xylanolytic; whereas some of these including Chaetomium globosum, and a Chaetomium sp. were found to be ligninolytic as well. These isolated fungi possessed the ability to tolerate highly alkaline pH's in their growth media. Some previous investigators have reported the influence of alkaline pH conditions on the growth of some fungi. By inoculating A. fumigatus on mineral salts solution adjusted to pH values ranging from pH3-9, Mills and Eggins (1970) reported that this fungus grew better at neutral or slightly alkaline pH values than at acid pH values. Lie and March (1968) inoculated the fungi A. flavus and A. parasiticus separately on acid curd (from skimmed milk mixed and blended with water); the pH of the substrate was adjusted to pH values ranging from 1.0-11.35 using hydrochloric acid and sodium hydroxide and from 1.8-10.70 using lactic acid and ammonium hydroxide. By measuring the pH of the inoculated substrates after 21 days' incubation at ambient temperature, they found that the pH values of nearly all the samples approached neutrality. Koburger (1972) without identifying up to species level has reported the isolation of a number of moulds from 25 retail food samples by inoculating onto antibiotic-containing potato dextrose agar, adjusted to pH values ranging from pH 7.0-10.0. After measuring the pH values of the growth media he found that the largest count of fungi was recorded at pH 8.00.

When the second set of agar media was used, most of the isolated fungi were the ones which were already reported from the first set of media used for isolation. In addition, a number of fungi not previously reported from the first set of media were also isolated and some of the most common ones were Aureobasidium pullulans, Aspergillus flavus, A.

versicolor, Botrytis sp. and Penicillium spp.

The abundant isolation of some of those fungi were also noticed which were reported occasionally from the first set of media. As mentioned before the growth conditions provided from the first set of media were not quite suitable for a number of fungi, despite the fungi being resistant to alkaline pH ranges and growing on cellulose agar media. Significantly, when pieces of straw treated with aqueous ammonia-added medium was inoculated on to cellulose agar plates, the appearance of these fungi, including Alternaria tenuis, Alternaria sp., Aspergillus spp., Cephalosporium sp. and Fusarium spp., were seen quite commonly. Abundant isolation of Aspergillus fumigatus, Chaetomium globosum, Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp. was also noticed after the incoulation of aqueous ammonia treated straw pieces onto cellulose agar.

After the isolation of fungi the pH of both sets of media were measured. It was found thatmost of the media from both sets still had an alkaline pH level, but a slightly higher alkaline pH range was recorded from the media supplemented with different concentrations of aqueous ammonia (1, 0.5, 0.25, 0.125 and 0.0625%). This suggests that ammonium ions were presumably acting as the buffering agent for maintaining the pH of the media during fungal isolation.

It was noticed, during the isolation programme from barley straw under alkaline conditions that Coprinus cinereus was the most common fungus isolated. This suggested the possibility of its isolation from other plant materials. An attempt was made to isolate Coprini from a range of vegetation collected from the urban and suburban areas of Birmingham.

Of the fourteen types of vegetation used, Coprini were isolated from ten of them. Five of these isolates were C. cinereus, three were C.lagopus and the remainder were not identified to species level.

Coprini have been isolated from the dung of herbivores by several workers - Carter, (1959); Webster, 1964; Hudson, (1972) recorded that as part of the coprophilous succession. Rege (1927) reported the isolation of Coprinus sp. from a heap of horse manure, whereas Fries (1955) collected some species of Coprini from stable manure.

As far as the presence of Coprini on plant materials are concerned, Eastwood (1952) reported the isolation of C.lagopus from a barley straw compost and Chang and Hudson (1969) reported the isolation of several Coprinus species from wheat straw compost. C.cinereus was isolated by many workers at the B.I.C. from varieties of wheat and barley straw (Seal, 1973; McShane, 1976 and Penn, 1977).

The dispersal of the spores of the Coprini was reported by Buller (1909) to be mainly anemophilous. This would result in spores occurring on many different surfaces. Very little isolation work has been done on the Coprini and most of this focused on cereal straws. Warcup (1957) reported the isolation of 210 fungal species when he studied the occurrence and activity of fungi in a wheat field-soil. Although he noticed the appearance of several species of Basidiomycetes by employing the hyphal isolation method, but he did not report the isolation of any coprinus sp. Warcup (1951) has also reported the isolation of 148 fungal species from soil samples collected from fine natural grasslands (which showed a wide variation in pH reaction, i.e. from a shallow, highly alkaline

horizon to a deep, highly acidic podsol), but again failed to report the isolation of any *Coprinus* sp. Fries (1955) reported that most of *Coprini* can grow preferentially under alkaline pH conditions. Thus it is possible that Warcup failed to isolate any *Coprinus* species because of the acidic isolation media he used.

Sagara (1975) has recently reported the presence of a number of fungi including several *Coprini* from highly alkaline soils which were previously treated with urea or aqueous ammonia. This suggests the possibility of the isolation of *Coprini* from such types of ammonia treated plant materials. It is possible that ammonia plays a vital role in the isolation of *Coprini*, as Fries (1955) reported that all her tested *Coprini* were preferentially utilizing ammonium ions as the sole nitrogen source instead of using nitrate.

To create alkaline conditions, 1% v/v aqueous ammonia was used to isolate *Coprini* from different vegetation. The highly alkaline conditions produced by the addition of ammonia suppressed the growth of other fungi on the vegetation and allowed the *Coprini* to be isolated.

A range of incubation temperatures was employed for the isolation of mesophilic, thermophilic or thermotolerant fungi from barley straw under alkaline pH conditions. Where alkaline pH condition was provided by the addition of either aqueous ammonia or by the use of 1N sodium hydroxide (NaOH). When an incubation temperature between 20-35°C was employed, then it was observed that most of the fungi isolated were the ones which were previously recorded in Chapter 2, where the growth condition was particularly suitable for the appearance of mesophilic fungi. However, most of these mesophilic fungi were

recognized as cellulolytic and xylanolytic including Alternaria tenuis, Alternaria sp., Aspergillus niger, A. flavus, A. versicolor, Aspergillus sp., Aureobasidium pullulans, Botrytis cineria, Chaetomium globosum, Coprinus cinereus, Fusarium sp., Gliocladium sp., Scopulariopsis brevicaulis, S. chartarum, Scouplariopsis sp. and Streptomyces sp. The occasional appearance of Botryotrichum piluliferum (1), Gliocladium roseum (2) and Stachybotry atra (3) are also noticed, but the appearance of the first two fungi were not recorded below 30°C incubation temperature. It is important to note that, out of these commonly isolated, mesophilic fungi, the most abundant appearance was noticed by Chaetomium globosum, Coprinus cinereus and Scopulariopsis brevicaulis, on all types of growth media used, in various isolation techniques which were reported in this investigation. The reason should be their abilities to tolerate highly alkaline pH conditions in their growth environments.

Most of the mesophilic fungi outlined above have been reported by many workers as the permanent or common flora on cereal products and by-products - particularly straws. Chirstensen (1955) reported the presence of Alternaria sp., Cladosporium sp. and Fusarium sp. as being abundant on barley seed before the seed matured. Webster (1956, 1957) reported the colonisation of Cladosporium herbarium, Aureobasidium pullulans and Alternaria tenuis on straw prior to the process of composting. Flannigan (1969) has also indicated the isolation of a number of fungi from dried barley grain including Alternaria tenuis, Aspergillus glaucus, Aureobasidium sp. Aureobasidium pullulans, Botrytis cineria, Cladosporium spp. Fusarium avenaceum. Likewise, Ogundana (1977) studied the

subepidermal presence of fungal mycelium in wheat grains just before harvest and subsequently in storage; he reported the isolation of *Alternaria alternata*, *Aspergillus flavus*, *Cladosporium cladosporioides* and *Fusarium semitecum*.

By employing various isolation techniques at a range of incubation temperatures, a number of thermophilic or thermotolerant fungi were also isolated, including *Aspergillus fumigatus*, *Chaetomium globosum*, *Cephalosporium* sp., *Humicola* sp., *Mucor* sp., *Myriococcum albomyces*, *Paecilomyces* sp. *Rhizopus* sp. and *Streptomyces* sp. Among these isolates the most common ones were found to be *Aspergillus fumigatus* and *Cephalosporium* sp., which appeared at all incubation temperatures, i.e. 20-50°C used for isolation.

Althought the abundant appearance of *A. fumigatus* was noticed on most of the agar media used, its colony diameter was seen to be smaller on these media. This shows that this fungus can tolerate highly alkaline pH conditions and a range of incubation temperatures, but could not show good growth under these circumstances. Some of the previous investigators have also reported the isolation of *A. fumigatus* at higher incubation temperatures. Likewise, Eastwood (1952) investigated the isolation of thermophilic fungi from lawn mowings and cut barley straw composts and reported the isolation of this fungus.

Chang and Hudson (1969) recorded the presence of *A. fumigatus*, when they had isolated fungal flora from wheat straw compost at higher incubation temperatures. Flannigan (1969) mentioned the presence of *A. fumigatus*, inoculating dried barley grains on agar plates incubated for the isolation of thermotolerant fungi. An abundant appearance of

this fungus was also noticed by Malik (1970), from most of the methods he used for the isolation of cellulolytic fungi, by using soil enrichment techniques.

McShane (1976) at the Biodeterioration Information Centre (B.I.C.) has also reported the abundant appearance of A.fumigatus, when he isolated the fungi from wheat straw during his investigation on the colonisation of non-sterile wheat straw fungi. He has also sampled the atmosphere within the B.I.C. on several occasions and all times reported the isolation of this fungus. He suggested that the high frequency of colonisation of A. fumigatus may well be a characteristic of the B.I.C. Laboratories. This fungus has been shown to be prevalent in the atmosphere and the straw kept within the confines, which may be heavily exposed to continual showers of spores of A. fumigatus. McShane justified his hypothesis by using freshly cut straw, which had not been stored in the B.I.C. premises, which was then inoculated for fungal isolation and observed that A.fumigatus was not isolated by this technique. A recent investigator at the B.I.C. (Penn, 1977), has also reported the abundant isolation of this fungus when he isolated fungi from barley straw which could presumably justify the hypothesis made by Mcshane that the B.I.C. laboratories atmosphere is contaminated with this fungus. In addition, the author also assumed the same reasons for the abundant isolation of A.fumigatus when he studied the fungal isolation from barley straw which was stored in the B.I.C. premises.

The fungus Cephalosporium sp. was also found to be the most abundant isolate which had appeared at the range of incubation temperatures used, but its isolation was

occasional on growth media supplemented with higher percentages of aqueous ammonia - particularly 1%, 0.5. and 0.25%. However, the establishment of this fungus on straw and grains was already reported by a number of investigators including Bruehl and Lai, (1966); Flannigan (1969) and Ogunada, (1977).

After isolating a number of fungi at a range of incubation temperatures, twelve of these isolates were further selected for measuring their growth activities under the influence of higher incubation temperatures, i.e. at 30°C, 35°C, 40°C, 45°C and 50°C. After measuring their mycelial growth, on YPSS agar medium, it was found that most of the fungi including A. fumigatus, Chaetomium sp., Mucor sp., Rhizopus sp. show their maximum colony diameters at 35°C, whereas Cephalosporium sp., Humicola sp. and Myriococcum albomyces at 40°C.

According to Cooney and Emersion (1964) the temperature limits for the growth of Myriococcum albomyces range from about 26°C-57°C, with the optimum falling between 37°C and 42°C, but the most rapid development took place at 40°C. On the other hand, no growth was seen at 25°C. Botryotrichum piluliferum and Paecilomyces sp. showed their maximum growth at 30°C. Coprinus cinereus showed its maximum growth at both 30°C and 35°C; but no fruiting body of this fungus was seen on the latter incubation temperature. Rege (1927) had also observed the extensive mycelial growth of a Coprinus sp at an incubation temperature of 35°C without seeing the production of fruting body.

Chapter 5 assessed the potential of commonest fungi to utilize various carbon sources including cellulose, hemi-

cellulose, ball-milled straw, phenolic compounds, i.e. gallic acid, tannic acid and lignins (lignosulphonate, Indulin A-R, and lignin prepared by 72% $H2SO_4$ method) which were added in their growth media. The purpose of this study was to discover a possible correlation between in vitro utilization of various carbon sources and in vivo decomposition of straw components when the fungi were actually colonised on straw. Nevertheless, the time factor did not allow an investigation of the biochemical analysis of straw components, after decomposition with colonised fungi.

By assessing the abilities of a number of selected fungi to utilize various carbon sources, it was found that some of the isolates were showing their extensive growth on the media supplemented with these substrates. These fungi were found to be Aspergillus fumigatus, Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus.

Many investigators (Day, Pelczar and Gottlieb, 1950; Siu, 1951; Gascoigne and Gascoigne, 1960; Norkrans, 1963; Chang and Hudson, 1967; Domsch and Gams, 1969; Flannigan, 1969 and Ghose, 1969) have reported the utilization of different carbon sources by a number of fungi. Some of these authors have also emphasised the importance of pH when studying the utilization of carbohydrates and lignocellulose complexes by fungi. Ghose (1969) suggested that the hydrogen ion concentration plays a key role in microbiological systems involving growth, enzyme systems and product formation. He further suggested that the enzyme synthesis systems by micro-organisms may undergo irreversible change if held at a pH value above or below its optimum.

Considering the cellulolytic activity of fungi Siu,

(1951) and Gascoigne and Gascoigne (1960) reported that fungal cellulases have a wide pH tolerance range, with optima from pH 3.0 (Hydnum henningsii) to pH 9.0 (Gliomastix convoluta). Thomas (1955) reported the pH optima for cellulase activity of Stachybotrys atra from pH 6.5 to 8.0, depending upon the substrate and method of assay used. Sharp and Eggins (1970) studied the influence of pH on the cellulolytic activity of fungi and reported that Fusarium sp has shown its cellulolytic activity throughout the pH range 3.7-8.6. Mills (1973) investigated the potential of twelve thermophilic fungi to degrade cellulose at different pH levels. He found that six of these fungi showed cellulase activity in the alkaline region. He concluded by suggesting that the fungi which can elaborate their cellulase system at alkaline pH values are involved in cellulose degradation with compost town waste.

Present studies also showed the role of a number of fungi to utilize different carbon sources including cellulose, xylan and ball-milled straw on the media adjusted under alkaline pH levels. whereas the growth media added with the substrate used for assessing the ligninolytic activity of fungi were not adjusted under alkaline pH level as it was assumed that the alkaline pH could inactivate the reactivity of phenolic substrates.

By taking into account a number of fungi including Aspergillus fumigatus, Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus who play a very important part in the utilization of different carbon sources added in their growth media, A.fumigatus showed growth on all different carbon sources added into its growth media. Nevertheless, its growth was considerably slower on agar plates in

comparison with the other three types of fungi. Its cellulose and xylan clearing activities were recorded as very strong - apart from the lesser clearing ability shown on ball-milled straw supplemented agar medium. As these media were adjusted by alkaline pH levels, one could suggest that although this fungus could show growth under alkaline conditions, its good growth may be possible at neutral or slightly acidic pH levels.

Ligninolytic activity of A. fumigatus was also examined on the scale of Bavendamm's polyphenoloxidase test (Bavendamm, 1928), but a negative reaction was recorded on both gallic acid and tannic acid media (added with malt extract agar). Its growth was seen on these media and also on the media containing different types of lignins added as the sole carbon sources. Here it could be assumed that the growth on the phenolic acid media may be due to the presence of malt extract which had possibly provided the easily available carbon sources to this fungus, instead of decomposing lignin by producing extracellular enzyme-polyphenoloxidase (Fahraeus, 1949).

If one could consider Bavendamm's scale as the positive criterion for assessing the ligninolytic activity of fungi, then it would be rather difficult to provide possible reasons for the growth of this fungus, on media where lignins were the sole sources of carbon.

Kirk and Kelman (1965) were also faced with similar situations where they found that a brown-rot fungus Poria monticola showed Bavendamm's test as negative but found it to be slightly active against another phenolic substrate,

cathecol. In addition, a recent investigation reported by Kirk (1975) also showed that another brown-rot fungus *Lenzites trabea*, which is also classed as negative by Bavendamm's test (Nobles, 1948) shows a very high demethyl ation in phenolic structural elements when this fungus attacked lignin.

After seeing the results recorded for the utilization of various carbon sources by *Coprinus cinereus*, it was found that it had extensive growth, with the formation of a thick white cottony mycelial mat and the formation of fruiting bodies on agar media with either added cellulose, hemicellulose or ball-milled straw. In addition a very strong clearing activity of this fungus was shown on these substrates added to the agar media.

The ligninolytic activity of this fungus was not recorded either on Bavendamm's test scale or by seeing its growth on different types of media supplemented with different lginins.

It has been mentioned on several occasions in this thesis that most of the *Coprinus* species - including *C. cinereus*, grow preferentially under an alkaline pH condition. Whereas in the present study, attempts were made to assess its ligninolytic activity by using acid pH media. Presumably this could be one of the possible reasons for the failure of this fungus to show any ligninolytic activity or growth, either on gallic acid, tannic acid added media or media supplemented with lignins.

Ligninolytic activities of some of the *Coprinus* species was reported by some workers. Waksman (1931) reported that *C. fimetarius*, *C. curtus*, *C.ephemerus*, *C.tardus* and *C.radians* are capable of decomposing lignin. He further reported that

when C. radians was inoculated onto raw horse manure it removed about 22 per cent of lignin of the total and 70 per cent of the cellulose in 51 days. Fries (1955) reported that some of her isolated Coprini were found to have ligninolytic activity by showing reactions towards the enzyme ligninase. Chang and Yee (1977) reported in a comparative study, of the physiology of Volvariella volvacae and Coprinus cinereus, that the latter fungus has shown a little growth on lignin added media, whereas much aerial mycelium and fructification of this fungus was shown on all the tested carbohydrates. Work is still in progress at the B.I.C. for studying the ligninolytic activity of Coprini - particularly Coprinus cinereus, but a fruitful result is still awaited. One could assume under these conditions that there may be a possibility of in vivo decomposition of lignin - particularly lignocellulosic complexes in straw. However, there is still need to improve the techniques for assessing ligninolytic activity of this fungus by employing both invivo and invitro examination methods. Recently, techniques have been advanced by some workers for measuring invivo (growing plant) lignin degradation using carbon-14-labelled material. These techniques are sensitive and specific and are particularly useful for quantitative examination of lignin biodegradation and for screening organisms, especially fungi for this ability. (Kirk, Connors, Blean, Hackett and Zeikus, 1975; Rosenber, 1978).

Positive results were recorded for Botryotrichum piluliferum and Chaetomium globosum by noticing good utilization of different carbon sources, by seeing extensive mycelial growth and very strong clearing on agar media, either

supplemented with cellulose, Xylan or ball-milled straw. When these fungi were tested for their ligninolytic activities wide darkened zones around their mycelial mats were seen. These zones showed their Bavendamm's test as positive which is indicative of their ligninolytic abilities. As good growth of these fungi were recorded in liquid and on solid media supplemented with different types of lignins, which justified their extracellular enzyme-polyphenoloxidase producing abilities.

All these characteristics shown by both Botryotrichum piluliferum and Chaetomium globosum suggests that there may be a possibility of high percentage weight loss in ligno-cellulosic components of straw, when these fungi were established and had colonised this substrate.

A number of other isolated fungi such as Alternaria tenuis, Alternaria sp., Aspergillus flavus, A. versicolor, Aspergillus sp., Botrytis cineria, Chaetomium sp., Gliocladium roseum . Fusarium sp., Myriococcum albomyces and Stachybotrys atra have also shown good utilization of different carbon sources. Some have also shown growth on phenolic acid and lignin added agar media. However, their growth and clearing on various media supplemented with various carbon sources were not as active and strong as reported for A. fumigatus, Botryotrichum piluliferum, Chaetomium globosum and Coprinus cinereus.

One of the important characteristics of these four fungi was noticed by observing their mycelial growth on carbon sourses containing (i.e. on 1% cellulose or 1% xylan) media, whose pH was adjusted to three different alkaline pH levels, i.e. pH 7.5, 8.5 and 9.5 (using sorensen phosphate buffer

solutions). This has suggested the idea that if one could inoculate straw with these fungi under alkaline pH conditions, then there may be a good chance for decomposition of straw components and less fear of contamination from un-inoculated fungi when non-sterile conditions prevail.

Thirteen different types of isolates were selected from the more commonly isolated fungi to study their colonisation on barley straw. The selection was made on their abilities to utilize various carbon sources, which were added to the growth media.

Fungal colonisation studies on straw recorded that when both sterile and non-sterile techniques were used, the most active substrate colonisers were found to be *Botryotrichum piluliferum*, *Chaetomium globosum*, *Coprinus cinereus*, *Gliocladium roseum* *Humicola* sp., *Myriococcum albomyces* and *Scopulariopsis brevicaulis*. These fungi showed rapid colonisation after inoculation on straw, impregnated with either L-asparagine (0.1% W/V per litre medium) or aqueous ammonia (1% or 0.5% V/V per litre medium) as the nitrogen sources added to the medium. It was noticed from other isolates such as *Alternaria tenuis*, *Aspergillus fumigatus*, *Cephalosporium* sp., *Fusarium* sp. and *Stachybotrys atra*, that their substrate colonisation was very poor or negligible in the flasks containing aqueous ammonia in the media. However, a better substrate colonisation was noticed by these fungi when the medium was supplemented with L-asperagine as the nitrogen source.

After fungal colonisation on straw (by incubating for an appropriate period of time) a final pH measurement was made on both types of the above mentioned media, by collecting liquid samples from the flasks. It was found that the samples

collected from most of the flasks were still at an alkaline pH level. Nevertheless, a slightly higher alkaline pH range was recorded from the media supplemented with aqueous ammonia, the one added with L. asparagine. The pH measurements indicate that all the inoculated fungi on straw can tolerate a range of alkaline pH conditions during their substrate colonisation.

It is assumed that the fungi that fail to colonise straw under such conditions because of their inability to utilize aqueous ammonia as the nitrogen source. However, another possibility to be borne in mind is that the presence of a high concentration of ammonium ions (1% or 0.5% V/V) in the media could cause an inhibitory effect on the growth of these fungi. (Nevertheless, no attempt was made to investigate such a hypothesis). This shows that when non-sterile conditions prevailed in a semi-solid fermentation of straw by inoculating it with a particular fungus which show rapid growth and a tolerance of high alkaline pH conditions (brought about by the use of aqueous ammonia), then the contamination with other fungi would be rare because of the selectivity of the media. In other words, un-inoculated fungi would only be able to colonize the substrate if they could tolerate the growth conditions. It was noticed in the present work that when non-sterile conditions prevail for fungal colonisation then a lesser number of un-inoculated fungi were seen appearing on the straw soaked in a media supplemented with aqueous ammonia whereas a greater number of contaminants were seen on straw supplemented with L-asparagine as a nitrogen source. However, some of the most common un-inoculated fungi seen appearing on the straw (which was inoculated with an initial fungus without autoclaving) were recorded as *Aspergillus fumigatus*,

Chaetomium globosum, Coprinus cinereus, Scopulariopsis brevicaulis and Streptomyces sp.

Previous chapters concerned with isolation showed that these fungi were isolated in abundance from straw under high alkaline pH conditions. This shows these fungi to be the established flora on the barley straw variety used in this investigation.

Colonisation on barley straw under sterile conditions was found to be the most rapid by Chaetomium globosum, Coprinus cinereus and Scopulariopsis brevicaulis. As they show extensive mycelial growth and rapid coverage of the entire surface area on the straw within an incubation period of ten days.

When non-sterile conditions were used, then the flask (inoculated with C. cinereus and containing a liquid media supplemented with 1 or 0.5% aqueous ammonia and straw) showed extensive mycelial growth of C. cinereus. Two of the un-inoculated fungi, Aspergillus sp. and Chaetomium sp. were seen from the flasks containing L-asparagine supplemented medium. This was confirmed by microscopic examination of the samples of straw pieces collected from flasks and when necessary cultured on agar plates.

Straw when initially inoculated with Chaetomium globosum show the appearance of un-inoculated fungi such as Coprinus cinereus and Scopulariopsis sp. When the media used was supplemented with 1% (V/V) aqueous ammonia or 0.1% (W/V) L-asparagine, there was no extensive growth of this fungus in comparison with straw treated with 0.5% (V/V) aqueous ammonia in the medium. Both macroscopic and microscopic examination of the straw showed no contamination. Here it is assumed

that the heavy growth of C. globosum had suppressed the appearance of other fungi. Likewise Penn (1977) also reported the rapid colonisation of the straw by this fungus when the same concentration of aqueous ammonia as mentioned above (0.5%) was added in the growth medium.

Similar results were also recorded for colonisation of Scopulariopsis brevicaulis on straw. Though no other fungi were seen in the flasks marked for 1% aqueous ammonia, the presence of Aspergillus sp., Chaetomium sp. and Streptomyces sp. was seen in the flasks marked for 0.5% (V/V) aqueous ammonia or 0.1% (W/V) L-asparagine.

By reviewing all the above outlined detail for fungal colonisation under non-sterile conditions, it was suggested that Coprinus cinereus was more capable of suppressing the appearance of most of the un-inoculated fungi were used as an inoculum for the semi-solid fermentation on straw.

The fungus Botryotrichum piluliferum was not reported abundantly in any of the techniques used for fungal isolation. Nor was this fungus reported to be among any of the un-inoculated fungi that appeared on the straw. Despite this fact, rapid colonisation of the straw was shown by this fungus in the flasks added with either type of nitrogen source. Similar characteristics as those mentioned for Coprinus cinereus were noticed for this fungus in both sterile and non sterile conditions. The presence of Aspergillus sp was only seen in the flasks added with L-asparagine, when the fungus was inoculated on straw under non-sterile conditions.

Cooney and Emerson (1964) have suggested that the thermophilic fungi play an important role in the decomposition of certain plant materials. According to their suggestion, some of the isolated fungi were selected for the study

of colonisation on straw under the influence of a range of higher incubation temperatures and by running both sterile and non-sterile conditions.

When both sterile and non-sterile conditions were used for colonisation of straw by four different types of fungi: A. fumigatus, Botryotrichum piluliferum, Coprinus cinereus and Myriococcum albomyces, it was found that the first and last fungi showed their growth at all the incubation temperatures used (35°C, 40°C, 45°C and 50°C) whereas the second and third fungi grew up to 45°C only though their growth rates were gradually decreasing beyond 35°C. However, it was noticed that 35°C incubation temperature was the optimum for a rapid colonisation by all the four fungi inoculated on straw.

When non-sterile conditions were prevailing and the flasks were incubated between 35-50°C, then the occasional appearance of Aspergillus fumigatus and Cephalosporium sp. was noticed on straw initially inoculated with Botryotrichum piluliferum, Coprinus cinereus and Myriococcum albomyces

Nevertheless, no un-inoculated fungi was seen in the straw flasks inoculated with Myriococcum albomyces - when an incubation temperature of 40°C was used. Cooney and Emerson (1964) also reported a maximum mycelial growth of this fungus at this incubation temperature.

In conclusion, it can be quite easily said that the two types of isolated fungi Coprinus cinereus and Botryotrichum- piluliferum, have shown their distinctive features (in comparision with other isolates) in many respects. These include extensive growths under the influence of highly alkaline pH conditions and at a range of incubation temperatures; very

strong activities and luxurient growth on the media supplemented by different carbon sources; and rapid colonisation on straw under sterile and non-sterile conditions, the latter causing the suppression in the growth of un-inoculated fungi.

Finally it can be said that the straw after treatment with the above two species of fungi could be considered as an up-graded product for feeding ruminants. Preferably one could select <u>Botryotrichum</u> <u>piluliferum</u> instead of <u>Coprinus</u> <u>cinereus</u>, as the agent for up-grading straw, because of its ligninolytic activity which was reported (tentatively) in both my own and the work presented by Haider and Gams (1969). They reported that when this fungus was grown on straw, a good utilization of cellulose and lignin resulted in the formation of humic substrates.

SUGGESTIONS FOR FURTHER INVESTIGATIONS

During the investigation period, a number of ideas have been born in mind for the continuation of further research in this field. These ideas are summarised as follows:

1) It would be interesting to study the isolation of fungi from seasonal varieties of barley straw, by collecting samples from different regions of England and Wales. The pH conditions for fungal isolation should be maintained within acidic to alkaline pH levels in conjunction with a range of incubation temperatures. The colony diameters of each isolate should be measured under these growth conditions by using pure culture inoculation.

2) Work is needed for the isolation of lignino-cellulolytic bacteria and actinomycetes from straw. Moreover the decomposition of straw by these micro organisms should also be studied using a range of organic and inorganic nitrogen sources.

3) Attempts should be made for the isolation of Coprini from seasonal vegetation. Soil samples should be collected from plates of growing vegetation. A pH measurement should be made and finally the samples should be plated out for the isolation of Coprini.

4) There is a need to investigate the roles of various organic and inorganic nitrogen sources used either for the growth of fungi on nutrient medium or for the decomposition of straw by colonised fungi.

5) It would be helpful to investigate the effect of ammonium ions on the growth activities of isolated fungi. This could be possible by treating fungal spores with different concentrations of aqueous ammonia before inoculation onto growth medium to allow mycelial growth.

6) Some new advanced techniques should be employed for the investigation of the ligninolytic activity of fungi and the degradation of lignified plant materials - particularly straw. One such technique involves the use of a new enzyme (the cellobiose: quinone oxidoreductase) for assessment of ligninolytic activity in fungi. Alternatively the measurement of lignin degradation by fungi (after invivo decomposition of lignocellulosic materials by the action of fungi) using carbon-12-labelled material.

7) A biochemical analysis of straw after decomposition by the action of thermophilic or thermotolerant fungi (from those isolated in this investigation) is needed.

R E F E R E N C E S

REFERENCES

ABRAMS, E. (1950) Microbiological deterioration of cellulose during the first 72 hours of attack. Textile Research Journal 20 (2) 71-86.

A.C.A.H. (1973) Report on straw disposal

AGARWAL, P.N. VERMA, G.M., VERMA, R.K. and SAHGAL, D.D. (1963) Decomposition of cellulose by the fungi Chaetomium globosum: Part 1. Studies on enzyme activity. Indian Journal of Experimental Biology 1 (1) 46-50.

ALLSOPP, D. (1973) Some aspects of the colonisation and decay of fungicidally protected cotton textiles by soil fungi. Ph.D. Thesis, University of Aston in Birmingham

ADAMS, G.A. and LEDINGHAM, G.A. 1942) Biological decomposition of chemical lignin 1. Sulphite waste liquor. Canadian Journal of Research, 20C, 1-12.

ANDERSON, G.E. (1959) Induced mutants in Coprinus lagopus. Heredity, 13: 411-412.

ASCHAN, K. and NORKRANS, B. (1953) A study in the cellulolytic variation for wild types and mutants of Collybia Velutipes 1., Physiologia Plantarum 6: 564-583.

BAKER, A.J. and MILLET, M.A. (1975) Wood and wood-based residues in animal feeds. Cellulose Technology Research, A.C.S. Symposium series 10, American Chemical Society, Washington, D.C.

BARNETT, H.L. (1972) Illustrated genera of Imperfecti fungi. Third Edition. Burgess Company

BARRON, G.L. (1968) The genera of Hyphomycetes from soil. Williams and Wilkins, Baltimore.

BAVENDAMM, W. (1928) Uber das vorkommen und der Nachweis von oxydasen baeholzzerstorenden pilzen, Z. für pflanzenkrankh 38: 257-276.

BJORKMAN, A. (1957) Studies on finely divided wood Part 2. The properties of lignins extract with neutral solvents forms of wood and hardwood. Svensk Papperstidn, 60, 158.

BLOCHWITZ, A. (1914) Botryotrichum piluliferum Morphologie, Entwicklunsgeschichte, Physiologie, Okalogi. Anals Mycol 12, 315-334.

BORUT, S.H.Y. (1960) An ecological and physiological study on soil fungi of the Northern Neger (Israel). Bull. Res. Coun. Israel, 8D, 65-80.

BRIERLEY, W.B., JENSON, S.T. and BRIERLEY (1927) The quantitative study of soil fungi. Proc. and papers. 1st Int. Congr. Soil Sci. Commission, 3, 48.

BRUEHL, G.W. and LAI, P. (1966). Prior-colonization as a factor in the saprophytic survival of several fungi in wheat straw. Phytopythology 56. 766-768.

BULLER, A.H.R. (1909) Researches on fungi. Vol. 1, 1958. Hanfer Publishing Company, New York.

BULLER, A.H.R. (1924) Researches on fungi. Vol. III, 1958 Hanfer PUblishing Company, New York.

BULLER, A.H.R. (1931) Researches on fungi. Vol. lV, 1958. Hanfer Publishing Company, New York.

BULLER, A.H.R. (1933) Researches on fungi. Vol. V, 1958. Hanfer Publishing Company, New York.

BURGES, A. (1939) Soil fungi and root infection. Brotesia 8, 35. Fax 2

ARMONA, J.F. and GREENHALGH, J.F.D. (1973) The digestibility and acceptability to sheep of chopped or milled-barley straw soaked or sprayed with alkali. Journal of Agricultural Science, Cambridge 78: 477-485.

CARTER, S. (1959) Some investigations into the succession of fungi on rabbit dung. M.Sc. Thesis. University of London

CARTWRIGHT, K.st.G. (1934) Studies in the Physiology. J. Wiley & Sons, New York.

CASSELTINE, L.A. and LEWIS, D. (1967) Dilution of gene products in the cytoplasm of heterokaryons in Coprinus lagopus. Genet. Res. Camb. 9 (1): 63-71.

CHANDRA, S. and JACKSON, M.G. (1971). A study of various chemical treatments to remove lignin from coarse roughages and increase their digestibility. Journal of Agricultural Science, Cambridge, 77: 11-17.

CHANG, Y. (1967) The fungi of wheat straw composts. 11. Biochmical and Physiological studies. Transactions of the British Mycological Society, 50 (4): 667-677.

CHANG, Y. and HUDSON, H.J. (1967) The fungi of wheat straw compost. 1. Ecological studies. Transactions of the British Mycological Society, 50 (4): 649-666.

CHANG-HO, Y. and YEE, N.T. (1977) Comparative study of the physiology of Volvariella volvacea and Coprinus cinereus. Transactions of the British Mycological Society. 68 (2) 167-172.

CHESTER, C.G.C. (1940) A method of isolating soil fungi Transactions of the British Mycological Society 29: 354: 355.

CHESTER, C.G.C. (1948) A contribution to the study of fungi in soil. Transactions of the British Mycological Society, 30: 100-117.

COCHRANE, V.W. (1958) Physiology of fungi. New York: Wiley.

COONEY, D.G. and EMERSON, R. (1964) Thermophilic fungi. W.H. Freeman Publishing co., Sanfransisco.

COWAN, J.W. and LEWIS, D. (1966) Somatic recombination in the dikaryon of Coprinus lagopus. Genet. Res. Camb. 7 (2) 235-244.

COWLING, E.G. (1958) A review of literature on the enzymatic degradation of cellulose and wood. Forest Product Laboratory, U.S.A. Department of Agriculture Report No. 2116.

COWLING, E.B. (1961) Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. Tech. Bull., 1258, U.S. Dept. of Agricuture, Forest Service, Washington, 79 pp.

COWLING, E.B. (1963) Structural feature of cellulose that influence its susceptibility to enzymatic hydrolysis. In Advances in Enzymatic Hydrolysis of Cellulose and Related Materials. Pergamon Press, pp 1-32.

COWLING, E.B. and BROWN, W. (1963) Structure feature of cellulosic materials in relation to enxymatic hydrolysis in "Celluloses and their application". Advances in Chemistry Series. 95 pp, 152-187.

CRISAN, E.V. (1959) The isolation and identification of thermophilic fungi. M.Sc. Thesis. Purdue University.

DANIELS, J. (1961) Chaetomium piluliferum sp. Nov., The perfect state of Botryotrichum piluliferum. Transaction of the British Mycological Society. 44(1), 79-86.

DAVIDSON, R.W., CAMPBELL, W.A. and BLAISDELL, D.J. (1938) Differentiation of wood-decaying fungi by their reaction on gallic or tannic acid medium. Journal of Agricultural Research, 57, 683.

DODGE, B.O. (1912) Production of fertile hybrids in the ascomycete Neurospora. Journal of Agricultural Research, Vol. 36, pp 1-14.

DOMSCH, K.H. (1960) Das Pilzxpecktrum einer Bodenprobe. 1. Nachweis det Homogenitat. Arch. Mikrobiol., 35, 181-96.

DOMSCH, K.H. and GAMS, W. (1969) Variation and potential of a soil fungus population to decompose pectin, xylan and carboxymethyl-cellulose. Soil. Biol. Biochem. Vol. 1, pp 29-36. Pergamon Press. Printed in Great Britain.

DOSKACIL, J., (1948) Toxicity of buffer solutions. Acta Facult. Rev. Nat. University Carolina, 185:1.

DUNCAN, C.G. and ESLYN, W.E. (1966) Wood-decaying ascomycetes and fungi imperfecti. Mycologia 58, 642-645.

EASTWOOD, D.J. (1949) Fruiting of Coprinus lagopus in pure culture. Nature, London, 165, 324.

EASTWOOD, D.J. (1952) The fungus flora of composts. Transactions of the British Mycological Society, 35: 215-220.

EGGINS, H.O.W. and LLOYD, A.O. (1968) Cellulolytic fungi isolated by the screened substrate method. Experientia, 24, 749

EGGINS, H.O.W. and PUGH, G.J.F. (1962) Isolation of cellulose decomposing fungi from the soil. Nature. 193: 94-95.

ELLIAS, G.H., MATRONE, G and MAYNARD, L.A. (1946) a 72 percent H_2SO_4 method for the determination of lignin and its use in animal nutrition studies. Journal of Animal Science, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, New York.

ERIKSSON, K.E. (1974) Micro-organisms and enzymes and instruments in a new development in the wood industry. Norsk Skogindustri 5/74: 125.

ETHERIDGE, D.E. (1957) Differentiation of white and brown-rot fungi by an oxidase reaction. Nature 179: 921-922.

FAHRAEUS, G. (1949) On the oxidation of phenolic compounds by fungi. Ann. Roy. Agr. Coll., Sweden, 16, 618-629

FAHRAEUS, G. (1952) Formation of laccase by Polyporus versicolor in different culture media. Physiol. Plant. 5: 285-291.

FERGUS, C.L. (1964) Thermophilic and thermotolerant moulds and actinomycetes of mushroom compost during peak heating. Mycologia 56: 267-284.

FERGUS, C.L. (1969) The cellulolytic activity of thermophilic fungi and actinomycetes, Reprinted from: Mycologia, Vol. 61, No. 1 pp 120-129.

FISCHER, G. (1953) Untersuchungen uber den biologischen abbaudes Lignins durch Mikroorganisms. Arch. Mikrobiol. 18: 397-424.

FLANNIGAN, B. (1969) Microflora of dried barley grain. Transactions of tbe British Mycological Society, 53: (3) 371-379.

FLANNIGAN, B. (1970) Degradation of arabinoxylan and carboxymethyl cellulose by fungi isolated from barley kernels. Transactions of the British Mycological Society, 55 (2), 277-281.

FLANNIGAN, B. and SELLERS, P.N. (1972) Activities of thermophilic fungi from barley kernels against arabinoxylan and carboxymethyl cellulose. Transactions of the British Mycological Society, 58 (2) 338-341.

FORBES, R.S and DICKINSON, C.G. (1977) Effects of temperature, pH and nitrogen on cellulolytic activity of Fusarium avenaceum. Transactions of the British Mycological Society, 68 (2) 229-235.

FRAZ. H. and LOUB, W. (1959) Bodenbiologische untersuchungen an walddungungsversuchen. Zentbl ges forstw. 76, 129-62.

FRIES, L. (1945) Uber das wachstum einiger Coprinus Artenbie verschieden wasserstoffion-enkonzentrationen. Arkiv Botan. 32 (10): 1-8

FRIES, L. (1953) Factors promoting growth of Coprinus fimetarius under high temperature conditions. Physiol. Planterum 6: 551-563.

FRIES, L. (1955) Studies in the physiology of Coprinus I. Growth substrate, nitrogen and carbon requirements. Svensk. Bot. Tidskr. 49: 475-535.

FRIES, L.(1956) Studies in the physiology of Coprinus II. Influence of pH, metal factors and temperature. Svensk. Botanisk Tidskrift. 50 (1): 47-96

GARRETT, S.D. (1951) Ecological groups of soil fungi; a survey of substrate relations. New Phytologist, 50: 149-166.

GARRETT, S.D. (1962) Decomposition of cellulose in soil by Rhyzocotonia solani kuhn. Transactions of the British Mycological Society. 45: 115-120.

GARRETT, S.D. (1963) A comparison of cellulose decomposing ability in five fungi causing cereal foot-rots. Transactions of the British Mycological Society, 46: (4), 572-576.

GASCOIGNE, J.A. and GASCOIGNE, M.M. (1960) Biological degradation of cellulose. Butterworths.

GHOSE, T.K. (1969) Continuous enzymatic saccharification of cellulose with cultures filterates of Trichoderma viride QM6a Biotechnology and Bioengineering, 11, 239-261.

GILBERTSON, R.L. (1975) Gum Guaick in field tests for extracellular phenoloxidases of wood-rotting fungi and other basidiomycetes. U.S.A. For. Ser. Res. Paper 269.

GILMAN, J.C. (1957) A manual of soil fungi. Second edition. Iowa State Univ. Press, Iowa

GORDON, W.L. (1944) The occurence of Fusarium species in Canada, 1. Species of Fusarium isolated from farm samples of cereal seed in Manitoba. Canadian Journal of Research, C, 22, 282.

GULYAS, F. (1967) On the role played by several soil fungi in the microbiological decomposition of lignin. Agrokem. Talajtan 16: 137-150 (In Hungarian)

HAIDER, K. and DOMSCH, H. (1969) Abbau und umsetzung von lignifiziertem pflanzenmaterial durch mikroskospiche Bidenpilze. Arch Mikrobiol, 64, 338-348.

HAN, Y.W., CHEEKE, P.R., ANDERSON, A.W. and Lekprayoon, C. (1976) Growth of Aureobasidium pullulans on straw hydrolysate Appl. and Environ. Microb. 6, 799-802

HAN, U.W., DUNLAP, D.E. and CALLIHAN, C.D. (1971) Single cell protein from cellulosic wastes. Food Technoogy 25, 32-35, 56.

HARLEY, J.L. and WAID, J.S. (1955) A method for studying active mycelial on living roots and other substances in the soil. Transactions of the British Mycological Society, 38, 104-118.

HARPER, J.E.and Webster, J. (1964) An experimental anaylsis of the Coprophilous fungus succession. Transactions of the British Mycological Society, 47, 511-530.

HAZRA, A.K. BOSE, S.K. and GUNA, B.S. (1958) A rapid method of survey of cellulolytic power in fungi. Science culture 24: 1, 39-40.

HENDERSON, M.E.K. (1956) A study of the metabolism of phenolic compounds by soil fungi using spore suspensions. Journal of Microbiology. 14, 684

HENDERSON, M.E.K. (1963) Fungal metabolism of certain aromatic compounds related to lignin. Pure Applied Chemistry, 7. 589-602.

HENDERSON, M.E.K. and Farmer, V.C. (1955) Utilization by soil fungi of P-hydroxy-benzaldehyde, ferulic acid, Syringaldehyde and Vanillin. Journal of General Microbiology, 12, 37.

HESS, K. HAHL, H. and GUTTER, E. (1957) - Holloid Zeitschriff 55. 1. In Cowling and Brown (1969)

HESSELTINE, C.W., WHITEHILL, A.R., PIDACKS, C., TENHAGEN, M., BOHONS, M., HUTCHINGS, B.L. and WILLIAMS, J.H. (1953). Coprogen, a new growth factor present in dung required by Pilobolus. Mycologia, 45, 7-19

HIROI, T. and ERIKSSON, K.E. (1976) Microbiological degradation of lignin Part 1: Influence of cellulose on the degradation of lignins by the white-rot fungus Pleurotus ostreatus. Svensk Pappers, 79. (5), 162-166.

HIROI, T. and ERIKSSON, K.E. (1976) Microbiological degradation. Part 2. Influence of lignosulphonate of various molecular sizes of the white-rot fungus Pleurotus ostreatus. Savensk pappers, 79: (5), 162-166.

Hudson, H.J. (1972) Fungal saprophytism. Studies in biology No. 32. Edward Arnold (Publishers) Ltd.

HUDSON, H.J. and WEBSTER, J. (1958) Succession of fungi on decaying stems of Agropyron repens. Transactions of the British Mycological Society, 41: 165-177.

HUMPHREY, G.J. and SIGGERS, P.V. (1933) Temperature relation of wood-destroying fungi. Journal of Agricultural Research. Washington, D.C.

HUTCHKINSON and RICHARD, (1921) Artificial farmyard manure. J. Minist. Agric. 28, 398.

HYDE, H.A. and GALLEYMORE, H.B. (1951) The subepidermal fungi of cereal grains. II. The nature, identity and origin of the mycelium in wheat. Annals of Applied Biology, 38: 348-356.

INGOLD, C.T. (1953) Dispersal in fungi. Clarendon Press, Oxford.

ISHERWOOD, F.A. (1970) Polysaccharides of cell wall of fruit in "The Biochemistry of Fruits and Their Products" Academic Press. 36-44.

JENSEN, H.L. (1931) The fungus flora of the soil. Soil Science 31, 123-158.

JOHNSSON, G.T. and JONES, A.C. (1941) Data on the cultural characteristics of a species of Coprinus. Mycologia, XXXIII: 424.

KANEVSKAYA, I.G. (1966) Decomposition of methyl cellulose by soil fungi. Mikrobiologiya 36, 868-870.

KEMPLE, R.F.O. and WATLING, R. (1977) Coprinus workshop Bull. British Mycological Society, II (1): 24-27.

KIRK, T.K. CONNOR, W.J., BLEAM, R.D. HACKETT, W.F. and ZEIKUS, J.G. (1975) Preparation and microbial decomposition of synthetic $|C^{14}|$ lignins. Proc. Natl. Acad. U.S.A. 72: 2515-2519.

KIRK, T.K. (1975) Effect of a brown-rot fungus, Lenzites trabea, on lignin in spruce wood. Holzforsch, 29 (3): 99-107.

KIRK, T.K. and KELMAN, A. (1965) Lignin degradation as related to the phenoloxidases of selected wood-decaying basidiomycetes. Phytopathology, 55: 739-745

KNIEP, H. (1920) Neber morphologische und physiologisch Geschlechtsdifferenzierung (untersuchungen an Basidiomyzeten). Vehr. Phys. Med. Ges. Wurzburg, 46: 1-18

Koburger, J.A. (1972) "Fungi in Foods, IV Effect of pH of plating medium on counts" J.Milk.Fd. Technol 35 (1) 659-660

LARSEN, K. (1971) Bot. Tidsskr. 66, 1-32.

LAW, K. (1950) Phenoloxidases in some wood-rotting fungi. Ann. Botany, 14: 69-78

LEDINGHAM, G.A. and ADAMS, G.A. (1942) Biological decomposition of chemical lignin, II Studies on the decomposition of calcium lignosulphonate by wood-destroying and soil fungi. Can. J. Research, 20C, 13-27

LEVI, M.P., PRESTON, R.D. (1965) A chemical and microscopic examination of the action of the soft-rot fungus Chaetomium globosum on beechwood (Fagus sylv.) Holzforschyng, 19: 183-190.

LEVI, M.P. and COWLING, E.B. (1968) Role of nitrogen in wood deterioration. VII Physiological adaptation of wood-destroying and other fungi to substrates deficient in nitrogen. Phytopathology. 59: 460-468

LIE, J.L. and MARTH, E.H. (1968) Aflatoxin formation by Aspergillus flavus and Aspergillus parasiticus in a casein substrate at different pH values. J. Diary Sci., 51 (II) 1743-1747.

LIND, W. (1886) Mitteilungen uber einige neüe pathogens Schimmel pilze. Archive fur Experimentelle Pathologie und Pharmokolie. 21 269-298. (In Cooney and Emerson, 1964)

LINDEBERG, G. (1933) Decay of wood and growth of some hymenomycetes as effected by temperature. Phytopathology, 5 (5) 73.

LINDEBERG, G. (1944) Uber die physiologie ligninabbavender Boden-Hymenyzenten. Symbolae Botan. Uppsalienses, 8, 1-83

LINDEBERG, G. (1948) On the occurrence of polyphenoloxidases in soil-inhabiting basidiomycetes. Physiol Plantarum, 1: 196-205.

MALIK, K.A. (1970) Ecological studies on the biodeterioration of a model cellulose substrate by fungi. Ph.D. Thesis, University of Aston in Birmingham.

MALIK, K.A. and EGGINS, H.O.W. (1970) A perfusion technique to study the fungal ecology of cellulose deterioration. Transactions of the British Mycological Society. 54 (2): 289-301

MASSEE, G. (1906) A revision of the genus Coprini Annals of Botany, Vol. X. p 129

MASSEE, G. and SALMON, E.G. (1902) Researches on Coprophilous fungi II. Annals Bot. 16 57-93

MCBETH, I.G. (1916) Studies on the decomposition of cellulose in soils. Soil Science, 1: 437-487

McSHANE, C. (1976) The colonisation of non-sterile wheat straw compost by fungi. M.Phil. Thesis. University of Aston in Birmingham.

MERRIL, W., FRENCH, D.W. and HOSSFELD, R.L. (1965) Wood fiberboard studies, II. Effects of common moulds on physical and chemical properties of wood fiberboard. Tappi, 48, 470-474.

MICHELI, (1729) Nova Plantarum Genera, Florentiae, p.133. Table 73. (See Buller, 1924, Vol. III, (1958. Hanfer Pub. Co. N.York).

MIEHE, H. (1907) Die Selbsterhitzung des Heus. Eine biologische Studie, Jena: G. Fischer

MILLS, J. (1973) The biodegradation of certain components of town waste by thermophilic fungi. Ph.D. Thesis, University of Aston in Birmingham.

MILLS, J. and EGGINS, H.O.W. (1970) Growth of thermophilic fungi on oxidative product of polyethylene. International Biodeterioration Bulletin, 6, 13-17

MORRISON, F.B. (1959) Feed and Feeding. Morrison Publishing Co., Clinton, Iowa.

MORTON, A.G. and MACMILLAN, A. (1954) The assimilation of nitrogen from ammonium salts and nitrate by fungi. Journal of Experimental Botany, 5, 232-252.

MOUNCE, J. (1921) Homothallism and the production of fruit bodies by monosporous mycelia in the genus coprinus. Transactions of the British Mycological Society, 7: 256-269.

MUHTHERHALER, K. (1967) Ultra structure and formation of plant cell walls. Annual Review of Plant Physiology. 18: 1-24.

NICHOLSON, P.B. BOCOCK, K.L. and HEAL, O.W. (1966) Journal of Ecology, 54: 755-767.

NISIZAWA, K. (1963) Mode of action of cellulases. Journal of Fermentation Technology, 51, (4): 267-304

N.F.U. (1943) Report on the use and disposal of straw, by the working party on straw disposal, published by the

National Farmers Union.

NOBLES, M.K. (1948) Studies in forest pathology, VI. Identification of cultures of wood rotting fungi. Can. J. Res. 26 (C) 281-431

NOBLES, M.K. (1958) A rapid test for extracellular oxidase in cultures of wood-inhabiting hymenomycetes. Canadian Journal of Botany, 36: 91-99

NOBLES, M.K. (1964) Identification of cultures of wood inhabiting hymenomycetes. Can. Journ. of Bot. 43: 1097-1139

NORKRANS, B. (1963) Degradation of cellulose. Ann. Rev. Phytophathology, 1: 325-350

NORMAN, A.G. 1936) The biological decomposition of lignin. Science Progress, 30, 442-456.

O'CALLAGHAN, J.R. (1975) The waste products of agriculture J. Royal Soc. Arts. 123 (5223), 139-149.

OGUNDANA, S.K. (1977) Subepidermal fungi of wheat straw grains in India. Int. Biodeterioration Bulletin. (ISSN 0020-6164) 13 (1): 25-29.

OTTOW, J.C.G. (1972) Rose bengal as a selective aid in the isolation of fungi and actinomycetes from natural sources. Mycologia, 64 (2): 304-315.

PALMER, F.G. (1965) The feeding value of straw to ruminants. Agric. Devel. and Advi. Ser. Q. Rev. 21: 220-234

PENN, D.J. (1977) Some studies on the competative colonisation of cellulosic substrate by microorganisms. M.Phil Thesis. University of Aston in Birmingham .

PEITERSEN, N. (1975) Production of cellulase and protein from barley straw by Trichoderma viride. Biotechnology and Bioengineering, Vol. XVII, 361-374.

PEW, J.C. (1951) Structural aspects of the colour reaction of lignin with Phenols. Journal of Amer. Chem. Soc., 73: 1678-1685.

PHILIPS, M. (1934) Chemistry of lignin. Chem. Rev. 14: 103-107.

PINTO-LOPES, J. and ALMEDIA, (1971) "Coprinus lagopus". A confusing name as applied to several species. Portugalia Acta Biologica II, Series B, (3-4): 167-204.

PHOENIX, S.L., BILANSKI, W.K. and MOWAT, D.N. (1974) In vitro digestibility of barley straw treated with NaoH at elevated temperatures. Trans. Am. Soc. Ag., 17 (4) 780-782

PRESTON, A. and McLENNAN, E.I. (1948) The use of dyes in culture media for distinguishing brown and white wood-rotting fungi. Ann. Bott., 12: 53-64.

PRESTON, R.D. and CRONSHAW, J. (1958) Constitution of the fibrillar and non-fibrillar components of the walls of Valonia ventricosa Nature, 181: 248-250

PUGH, G.J.F., BLAKEMAN, J.P., JONES, G.M. and EGGINS, H.O.W. (1963) Studies on fungi in coastal soils, IV Cellulosic decomposing species in sand dunes. Transactions of the British Mycological Society, 40 (4) 505-571.

RAHN (1927) (See Rege 1927)

RANBY, B. (1969) Recent progress on the structure and morphology of cellulose in "Cellulases and their applications" Advances in Chemistry, Series 95, p 139-151.

RANBY, B and RYDHOLM, S.A. (1958) In "Polymer Processes - High Polymers" Inter. Science 10: 351.

RAPPER, K.R. and FENNELL, D.I. (1965) "The genus Aspergillus" Williams and Wilkins, Baltimore.

REESE, E.T. (1946) Aerobic decomposition of cellulose by microorganisms at temperatures above 40°C. Ph.D. Thesis Pennsylvania State College.

REESE, E.T. (1963) Advances in enzymatic hydrolysis of cellulose and related materials. Oxford, New York, Pergamon Press.

REGE, R.D. (1927) Biochemical decompositon of cellulosic materials with special reference to the action of fungi. Annals of Applied Biology, 14: 1-44.

RICHARDSONS and AMOORE (1927) (See Rege, 1927)

ROGERS, C.J., COLEMAN, E., SPINO, D.F. and PURCELL, T.C. (1972) Production of fungal protein from cellulose and waste cellulosics. Environmental Science and Technology, 5 (8) 715-718.

ROLLINS, M.L. and TRIPP (1961) The architecture of cellulose: A microscopic review. Forest Products Journal, 11 (II): 493-504.

ROSEMBERG, S.L. (1978) Cellulose and lignocellulose degradation by thermophilic and thermotolerant fungi. Offical organ of the mycological Society of America, Vol. LXX, No. 1; (70): 1-13.

ROSTRUP, O. (1916) Bidrag til Danmarks Svampeflora. 1 Danks. Bot. Ark. 2/5, 1-56.

SAGARA, N. (1975) Ammonia fungi - A chemicological grouping of terrestial fungi. Contribution from the Biological Laboratory, Kyoto University, Vol. 24. No. 4, p 205-276.

SARKANIN AND LUDWIG (1971) Occurrence, formation and structure of lignins. In Lignins. Wiley Inter. Science, New York.

SACCARDO, P.A.E. and Marshal, (1885) Champignons Coprophiles de Belgique. Bull. Soc. Roy. Bot. 24, 66-67.

SCHMIDT, E.G. PETERSON, W.H. and FRED, E.B. (1923) The destruction of pentosans by moulds and other micro-organisms. Soil Science, 15: 479-488.

SCALES, F.M. (1916) A new method of precipitating cellulose for cellulose agar. Centralblatt Bakt. Parasitenk. Abt. 11, 44: 661-663.

SEAL, K.J. (1973) Aspects of the fungal degradation of intensively produced farm animal manure. Ph.D. Thesis University of Aston in Birmingham

SEAL, K.J. and EGGINS, H.O.W. (1972) The role of micro-organisms in the Biodegradation of farm animal wastes with particular reference to intensively produced wastes. A review. International Biodeterioration Bulletin, 8 (3) 95-100.

SEAL, K.J. and EGGINS, H.O.W. (1976) The upgrading of agricultural wastes by thermophilic fungi. In "Food from agricultural waste" pages 58-77. Applied Science Publishers (1976)

SIU, R.H.G. (1951) Microbiological decomposition of cellulose. Reinhold Publishing Co.

SIU, R.G.H. and Sinden, J.W. (1951) Effect of pH, temperature and mineral nutrition on cellulolytic fungi. American Journal of Botany, 38: 284-290

SHARP, R.F. and EGGINS, H.O.W. (1970) The ecology of soft-rot fungi. I. Influence of pH. Internaional Biodeterioration Bulletin, 6, (2) 53-64

SORENSEN, H. (1953) Enzymatic hydrolysis of xylan. Nature, Lond. 172. 305-306.

SORENSEN, H. (1957) Microbiological decomposition of xylan. Acta Agric. Scan. Supplementum 1. 86 pp.

SOWDEN, F.J. and ATKINSON, H.J. (1949) Composition of certain soil organic matter fractions. Soil Sci. 68: 433.

SUKHENKO, F.T. and PODGAINAYA, E.S. (1951) Decomposition of proteins by enzymes of Fusarium avenaceum Biokhimyua, 16. 528-536.

TENNY and WAKSMAN (1929) Soil Science, 28: 55.

THORNTON, R.H. (1952) The screen immersion plate. A method of isolating soil micro-organisms. Research, 5: 190-191.

THOMAS, R. (1956) Fungal cellulases VII. Stachybotrys atra: Production and properties of the cellulolytic enzyme. Australian Journal of Biological Science 9: 159-183.

TRIBE, H.T. (1957) Ecology of microorganisms in soils as observed during their development upon buried cellulose film. In Microbial Ecology, 7th Symp. Soc. Gen. Microbial pp 287-298. Cambridge Univ. Press.

TRIBE, H.T. (1960) Aspects of decomposition of cellulose in Canadian soils. Can. Journ. Microbiol. 6, 317-323.

TUITE, F. and CHRISTENSEN, C.M. (1955) Grain storage studies XVI. Influence of storage conditions upon the fungus flora of barley seed. Cereal Chemistry. XXXII, No. 1, 1-11.

VAN SOET, P.J. and JONES, L.H.P. (1973) Laboratory methods for evaluating the energy value of feedstuffs. In "Feed Energy Sources for Livestock. Butterworths, 1976,

VAILLANT (1727) Botanicon Parisiense, Leide et. Amsterdam, p.73, Pl XII, Figures 11 and 12 (See in Buller, 1924, Vol. III, 1958)

WALKER, A.G. (1941) The colonisation of buried wheat straw by soil fungi with special reference to Fusarium culmorum

WAKSMAN, S.A. (1916) Soil fungi and their activities. Soil Science, 2: 103-155.

WAKSMAN, S.A. and SKINNER, C.E. (1926) Microorganisms concerned in the decomposition of cellulose in the soil. Journal of Bacteriology 12: 57-84.

WAKSMAN, S.A. (1927) Principal of soil microbiology. The William and Wilkins Co., Baltimore.

WAKSMAN, S.A. 1931) Decomposition of the various chemical constituents, etc. of complex plant materials by pure cultures of fungi and bacteria. Arch. Mikrobiol 2: 136-154.

WAKSMAN, S.A. and DIEHM, R.A. (1931) On the decomposition of hemicelluloses by microorganisms. I. Nature, occurrence, preparation and decomposition of hemicellulose. Soil Science, 32: 73-95.

WAKSMAN, S.A. UMBRIENT, W.W. and CORDON, T.C. (1939) Thermophilic actinomycetes and fungi in soils and in composts. Soil Science, 47, 37-61.

WALSH, J.H. and STEWART, C.S. (1969) A simple method for the assay of fungi. International Biodeterioration Bulletin, 5, (1): 15-20.

WARCUP, J.H. (1950) The soil plate method for isolation of fungi from soil. Nature 166: 117-118.

WARCUP, J.H. (1951) Transactions of the British Mycological Society, 34, 298.

WARCUP, J.H. (1955) Isolation of fungi from hyphae present in soil. Nature 175, 953.

WARCUP, J.H. (1957) Studies on the occurrence and activity of fungi in a wheat field soil. Transactions of the British Mycological Society, 40: 237-260.

WARCUP, J.H. (1960) Ecology of soil fungi. In "The ecology of soil fungi" Ed. by Parkinson, D. and Waid, J.S., Liverpool University Press.

WEBSTER, J. (1956) Succession of fungi on decaying cocksfoot culms I. Journal of Ecology, 44, 517-544.

WEBSTER, J. (1957) Succession of fungi on decaying cocksfoot culms II. Journal of Ecology, 45, 1-30.

WEBSTER, J. (1964) Culture studies on Hyopcrea and Trichoderma. 1. Comparison of the perfect states of H. gelatinosa, H. rufa and H. spec. I. Transactions of the British Mycological Society, 47, 75-96.

WEINGLING, R. (1938) Association effects of fungi. Botanical Review, 4: 475-596.

WESTERMARK, U. and ERIKSSON, K.E. (1974) Carbohydrate-dependent enzyme quionone reduction during lignin degradation. Polyporus versicolor Acta. Chem. Scand. B28 (2) 204-208.

WESTERMARK, U. and Eriksson, K.E. (1974) Cellulose: Quinone oxidoreductase, a new wood-decaying enzyme from white-rot fungi. Acta Chem. Scand. B28 (2) 209-214.

WHITE, W.L. MANDELS, G.R. and SIU, R.G.H. (1950) Fungi in relation to the degradation of woollen fabrics. Mycologia, 42, 199-223.

WOLING, (1927) (See Rege, 1927)

WOLPERT, F.S. (1924) Studies in the physiology of certain wood-destroying fungi in relation to the H-ion concentration of the media. Ann. Miss. Bot. Gard., 11: 43-97.

A P P E N D I C E S

Appendix

The compositions of the media used in this investigation are as follows:

(1) Eggins and Pugh cellulose agar:

KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	0.5 g
KCL	0.5 g
L-asparagine	0.5 g
Yeast extract	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2$	0.1 g
Agar	15 g
Cellulose (Whatman's) CFLL, ball-milled for suspension to 72 hours	250 ml of 4% to 1 litre
Distilled water	to 1 litre

(2) Xylan agar

KH_2PO_4	1.0 g
$(NH_4)_2SO_4$	0.5 g
KCL	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	0.1 g
Xylan (1% W/V per litre)	10 g
Distilled water	to 1 litre

(3) Ball-milled straw agar

Same as above, except xylan is replaced by ball-milled straw (250 ml of 4% suspension to 1 litre).

(4) Glucose-cellulose agar and Glucose-starch agar

See Page 17.

(5) Malt agar

Malt extract	20.0 g
Mycological peptone	5.0 g
Agar	15 g
Distilled water	to 1 litre

(6) Corn meal agar (Commercially prepared by Oxoid)

Cornmeal extract	2 g
(from 50 grams whole maize)	
Agar	15 g
Distilled water	to 1 litre

(7) Czapex dox agar (Commercially prepared by Oxoid)

Sodium nitrate	2.0 g
Potassium chloride	0.5 g
Magnesium glycerophosphate	0.5 g
Ferrous sulphate	0.01 g
Potassium sulphate	0.35 g
Sucrose	30 g
Oxoid agar No. 3	12.00 g
Distilled water	to 1 litre

(8a) Potato dextrose agar (Commercially prepared by Oxoid)

Potato extract (Oxoid L101)	4 g
Dextrose	20 g
Agar	15 g
Distilled water	to 1 litre

(8b) Potato-maltose agar (Kempal Watling, .977)

Cooked potatoes	200 g
Maltose	5 g
Agar	10 g
Distilled water	to 1 litre

(Squeeze the cooked and marked potatoe through muslin)

(9) YPSS agar

Difco powdered yeast extract	4.0 g
K2HPO4	1.0 g
$MgSO_4$.7H2O	0.5 g
Soluble starch	15.0 g
Agar	20.0 g
Water (¼ tap, ¾ distilled)	100 ml

(10a) Mineral salt agar media supplemented with a range of aqueous ammonia

KH2PO4	0.5 g
KCL	0.5 g
$MgSo_4$	0.2 g
Cal C/2	0.1 g
A range of aqueous ammonia	i) 10 ml (v/v per litre) for 1%

(10a) continued.

(for supplementing different percentages in the media)	ii) 5 ml (v/v per litre) for 0.5%
	iii) 2.5 ml (v/v per litre) for 0.25%
	iv) 1.25 ml (v/v per litre) for 0.125%
	v) 0.625 ml (v/v per litre) for 0.625%
Agar	15 g
Distilled water	to 1 litre.

(10b) <u>Mineral salt liquid media supplemented with a range of aqueous ammonia</u>

Same as mentioned above, except agar was replaced

(11) <u>Basal media</u>

See page 71.

(12) <u>Gallic acid and Tannic acid agar</u>

See page 71.

Media supplemented with different types of lignin (see pages 73 and 74.

<u>72% $h2SO_4$ lignin</u>

Prepared by the method described by Ellis, Matrone and Maynard (1946).